# COMPOSITIONS AND METHODS FOR TREATING CANCER USING CYTOTOXIC CD44 ANTIBODY IMMUNOCONJUGATES AND CHEMOTHERAPEUTIC AGENTS

5 **RELATED APPLICATIONS**

The priority benefit of EP 02 018 686.2, filed August 21, 2002 and U.S. Provisional Application No. 60/405,956, filed August 26, 2002 are hereby claimed, both of which are incorporated by reference herein.

10 **BACKGROUND**

The invention relates to the combined use of conjugates of antibodies with cytotoxic compounds and chemotherapeutic agents in cancer therapy, pharmaceutical compositions comprising such compounds and/or chemotherapeutic agents, and methods of cancer treatment.

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There have been numerous attempts to improve the efficacy of antineoplastic drugs by conjugating such drugs to antibodies against tumor-associated antigens in order to elevate local concentration of the drug by targeted delivery to the tumor. Many of these approaches have met limited success, and several reasons have been discussed in the

20 literature to explain the failure. For anticancer drugs acting stoichometrically, like e.g. doxorubicin or methotrexate, relatively high intracellular concentrations are necessary to exert the required cytotoxicity. These concentrations are thought to be difficult to achieve with many antibody-drug conjugates because of (a) insufficient potency of many common anticancer drugs, (b) low cell surface concentration of antigen targets, (c) inefficient

25 internalization of antigen-antibody complexes into the target cell, and (d) inefficient release of free drug from the conjugate inside the target cell (Chari, RVJ et al., Immunoconjugates containing novel maytansinoids: promising anticancer drugs. Cancer Research 52: 127-31, 1992).

30 Two of the aforementioned drawbacks, namely (a) and (d), have been adressed by the work of Chari and coworkers (Chari, RVJ et al., Immunoconjugates containing novel

maytansinoids: promising anticancer drugs. Cancer Research 52: 127-31, 1992; Liu, C et al., Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. Proc. Natl. Acad. Sci. U.S.A 93: 8618-23, 1996; U.S. Patent No. 5,208,020). They have developed antibody conjugates wherein the antibody is linked to a maytansinoid via a

5     disulfide linkage. Maytansines belong to the class of Ansa macrolide antibiotics, which derive from *Nocardia sp*. The maytansine ansamitocin P-3, produced by bacterial fermentation, is used as a precursor molecule to manufacture maytansinoid DM1. Maytansine and derivatives act as anti-mitotic agents (inhibitors of tubulin polymerization), similar as vincristine, but with markedly higher potency than vincristine

10     or other established chemotherapeutic agents (DM1 is toxic to cells *in vitro* at about $10^{-10}$M concentration). In contrast to the high cytotoxicity of free maytansinoid, the antibody conjugate has a toxicity which is several orders of magnitude lower on antigen-negative cells compared to antigen-positive cells. The linkage by disulfide bonding has the advantage that these bonds are readily cleaved inside the target cells by intracellular

15     glutathione, releasing highly toxic free drug. This approach has been applied to antibodies against tumor-associated antigens, for example the C242-DM1 conjugate (Liu, C et al., Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. Proc. Natl. Acad. Sci. U.S.A 93: 8618-23, 1996; Lambert, JM et al., Pharmacokinetics, in vivo stability, and toxicity of the Tumor-activated prodrug, C242-DM1, a novel colorectal

20     cancer agent. Proceedings of the American Association of Cancer Research 39: Abs 3550, 1998; Tolcher AW et al. SB-408075, A maytansinoid immunoconjugate directed to the C242 antigen: a phase I pharmacokinetic and biologic correlative study. Poster 11th Symp. on new drugs in cancer therapy (Nov 7-10, 2000 in Amsterdam), 2000), and HuN901-DM1 (Chari, RVJ et al., Dose-response of the anti-tumor effect of HUN901-DM1 against

25     human small cell lung cancer xenografts. Proceedings of the American Association of Cancer Research (April 1-5, 2000) 41:(April 1-5) Abs 4405, 2000). However, the application of these conjugates is restricted due to the limited expression of the respective target antigens. For example, the antigen recognized by N901 (CD56, N-CAM) is predominantly expressed by tumors of neuroendocrine origin, the expression of the C242

30     antigen (CanAg) is mostly limited to tumors derived from the GI tract.

There is, therefore, still the need to improve this approach by finding suitable tumor-associated antibodies with favorable antigen expression pattern, high and specific cell surface antigen concentration within the target tissue, and efficient internalization process transporting the antigen-complexed antibody conjugate into the cells.

CD44 is a protein which is expressed in several different isoforms on the surface of a wide variety of cell types. The smallest isoform, standard CD44 (CD44s), which is expressed by a variety of different cells, is thought to mediate cell attachment to extracellular matrix components and may transmit a co-stimulus in lymphocyte and monocyte activation. In contrast, expression of splice variants of CD44 which contain the domain v6 (CD44v6) in the extracellular region, is restricted to a subset of epithelia. The physiological role of CD44v6 is not yet fully understood.

CD44v6, as well as other variant exons (CD44v3, CD44v5, CD44v7/v8, CD44v10) has been shown to be a tumor-associated antigen with a favorable expression pattern in human tumors and normal tissues (Heider, K-H et al., Splice variants of the cell surface glycoprotein CD44 associated with metastatic tumor cells are expressed in normal tissues of humans and cynomolgus monkeys. Eur. J. Cancer 31A: 2385-2391, 1995; Heider, K-H et al., Characterization of a high affinity monoclonal antibody antibody specific for CD44v6 as candidate for immunotherapy of squamous cell carcinomas. Cancer Immunology Immunotherapy 43: 245-253, 1996; Dall et al., 1996; Beham-Schmid et al., 1998; Tempfer et al., 1998; Wagner et al., 1998) and has been subject to antibody-based diagnostic and therapeutic approaches, in particular radioimmunotherapy (RIT) of tumors (Verel et al., Int. J. Cancer 99: 396-402, 2002; Stromer et al., 2000, WO 95/33771, WO 97/21104).

However, a prerequisite for efficient killing of tumor cells by antibody maytansinoid conjugates is sufficient internalization of the target antigen. Only few data on the internalization of CD44 are available. Bazil and Horejsi reported that downregulation of CD44 on leukocytes upon stimulation with PMA is caused by shedding of the antigen rather than by internalization (Bazil, V. and Horejsi, V. Shedding of the CD44 adhesion

molecule from leukocytes induced by anti-CD44 monoclonal antibody simulating the effect of a natural receptor ligand. J. Immunol. 149 (3):747-753, 1992). Shedding of CD44 is also supported by several reports on soluble CD44 in the serum of tumor patients and normal individuals (Sliutz, G et al., Immunohistochemical and serological evaluation of

5   CD44 splice variants in human ovarian cancer. Br.J.Cancer 72: 1494-1497,, 1995; Guo et al., Potential use of soluble CD44 in serum as indicator of tumor burden and metastasis in patients with gastric or colon cancer. Cancer Res 54 (2): 422-426, 1994; Martin, S. et al., Soluble CD44 splice variants in metastasizing human breast cancer. Int. J. Cancer 74 (4): 443-445, 1997). In a recent paper by Aguiar et al. the amount of internalized CD44 on

10   matrix-intact chondrocytes was determined to be approximately 6% in 4 hours (Aguiar, DJ et al., Internalization of the hyaluronan receptor CD44 by chondrocytes. Exp.Cell.Res. 252: 292-302, 1999). Similar low levels of internalized CD44v6 on tumor cells were found in experiments performed by BIA. Taken together, these data suggest that CD44 receptors are more likely subject to shedding than to internalization, and thus CD44

15   specific antibodies are not to be regarded as suitable candidates for the maytansinoid conjugate approach. This has been supported by in vitro cell proliferation assays wherein $Ab_{CD44v6}$-DM1 showed only slightly elevated cytotoxicity against antigen-presenting cells as compared to cells lacking the antigen.

20   It now has been found that CD44 specific antibodies conjugated to highly cytotoxic drugs through a linker which is cleaved under intracellular conditions are very efficient tumor therapeutics in vivo. Thus, such compounds may be advantageously used in cancer therapy.

25   There is still the need for further improvements. For the antibody maytansinoid conjugates huN901-DM1 and C242-DM1, the combination of these conjugates with the taxanes paclitaxel or docetaxel has been suggested (WO 01/24763).

It has now been unexpectedly found that the combination of a conjugate consisting of a

30   CD44 specific antibody and a cytotoxic agent with a further chemotherapeutic agent shows synergistic effects.

## SUMMARY OF THE INVENTION

The invention relates to the combined use of conjugates of CD44 specific antibodies with cytotoxic compounds and chemotherapeutic agents in cancer therapy, pharmaceutical compositions comprising such compounds and/or chemotherapeutic agents, and methods of cancer treatment. Preferred conjugates contain maytansinoids as cytotoxic compounds, and preferred chemotherapeutic agents are taxanes, epothilones, and vinca alcaloids.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1:** In vitro cytotoxicity of BIWI 1. The antigen-positive cell lines A431 and FaDu and the antigen-negative cell line A549 were used.

**Figure 2:** Efficacy of BIWI 1 treatment in nude mice xenografted with A431 tumors. The average tumor volumes per group with standard deviations are shown, the treatment groups are indicated. The arrow indicates start of treatment (day 1).

**Figure 3:** Efficacy of BIWI 1 treatment in nude mice xenografted with FaDu tumors. The average tumor volumes per group with standard deviations are shown, the treatment groups are indicated. The arrow indicates start of treatment (day 1).

**Figure 4:** Efficacy of BIWI 1 treatment in nude mice xenografted with MDA-MB 453 tumors. The average tumor volumes per group with standard deviations are shown, the treatment groups are indicated. The arrows indicate the treatment days.

**Figure 5:** Tolerability of BIWI 1 treatment. The average body weight change of all treatment groups in the 2 investigated models is shown. Day 1: start of treatment.

**Figure 6:** Efficacy of BIWI 1 combination treatment with paclitaxel in nude mice xenografted with FaDu tumors. The average tumor volume per group with standard deviations is shown.

**Figure 7:** Efficacy of BIWI 1 combination treatment with paclitaxel in nude mice xenografted with FaDu tumors. The individual tumor volumes per group are shown.

**Figure 8:** BIWI 1 combination treatment with paclitaxel in nude mice xenografted with

5    FaDu tumors. Tolerability of treatment: The average body weight change of all treatment groups is shown. Day 1: start of treatment.

## DETAILED DESCRIPTION

In particular, the present invention relates to the use of a compound of formula

10    $A(LB)_n$ (Formula (I),

wherein

**A**    is an antibody molecule which is specific for CD44;

**L**    is a linker moiety;

**B**    is a compound which is toxic to cells; and

15    **n**    is a decimal number with n = 1 to 10

for the preparation of a pharmaceutical composition for the treatment of cancer, wherein said compound is used or is for use in combination with a further chemotherapeutic agent. In a further aspect, the present invention relates to a method of treatment of cancer,

20    wherein an effective amount of a compound of Formula (I), as defined herein, is administered to a patient in need thereof in combination with a further chemotherapeutic agent.

The antibody molecule **A** has a binding specificity for CD44, preferably variant CD44,

25    most preferably CD44v6.

The term „antibody molecule" shall encompass complete immunoglobulins as they are produced by lymphocytes and for example present in blood sera, monoclonal antibodies secreted by hybridoma cell lines, polypeptides produced by recombinant expression in host

30    cells which have the binding specificity of immunoglobulins or monoclonal antibodies, and molecules which have been derived from such immunoglobulins, monoclonal

antibodies, or polypeptides by further processing while retaining their binding specificity. In particular, the term „antibody molecule" includes complete immunoglobulins comprising two heavy chains and two light chains, fragments of such immunoglobulins like Fab, Fab', or F(ab)$_2$ fragments (Kreitman, RJ *et al.*, *Pseudomonas* exotoxin-based

5     immunotoxins containing the antibody LL2 or LL2-Fab' induce regression of subcutaneous human B-cell lymphoma in mice. Cancer Res. 53: 819-825, 1993), recombinantly produced polypeptides like chimeric, humanised or fully human antibodies (Breitling, F. and Duebel, S. Recombinant Antibodies. John Wiley, New York, 1999; Shin, S-U and Morrison S L. Production and properties of chimeric antibody molecules. Methods

10    Enzymol. 178: 459-476, 1989; Güssow D, and Seemann G. Humanization of monoclonal antibodies. Methods Enzymol. 203: 99-121, 1991, Winter, G et al., Making antibodies by phage display technology. Ann. Rev. Immunol. 12: 433-455, 1994, EP 0 239 400; EP 0 519 596; WO 90/07861 EP 0 368 684; EP 0 438 310; WO 92/07075; WO 92/22653; EP 0 680 040; EP 0 451 216), single chain antibodies (scFv, Johnson, S. and Bird, R E.

15    Construction of single-chain derivatives of monoclonal antibodies and their production in Escherichia coli. Methods Enzymol. 203: 88-98, 1991), multimeric antibodies (Kortt, AA et al., Dimeric and trimeric antibodies: high avidity scFvs for cancer targeting. Biomol. Eng. 18(3), 95-108, 2001) like diabodies, triabodies, or tetrabodies, and the like. Today, antibodies may also be produced without immunising a laboratory animal, e.g. by phage

20    display methods (Aujame, L. et al., High affinity human antibodies by phage display. Hum. Antibodies 8(4):155-68, 1997; U.S. Patent 5,885,793; U.S. Patent 5,969,108; U.S. Patent 6,300,064; U.S. Patent 6,248,516, U.S. Patent 6,291,158). Fully human antibodies may be produced using transgenic mice carrying functional human Ig genes (EP 0 438 474 ; EP 0 463 151; EP 0 546 073). From the aforementioned literature references, the expert

25    knows how to produce these types of antibody molecules, employing state of the art methods like automated peptide and nucleic acid synthesis, laboratory animal immunisation, hybridoma technologies, polymerase chain reaction (PCR), vector and expression technologies, host cell culture, and protein purification methods. In the following, the terms „antibody" and „antibody molecule" are used interchangeably.

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„Specific for CD44" shall mean that the antibody molecule has specific binding affinity for an epitope present in CD44. In a preferred embodiment, the antibody molecule of the invention has a binding specificity for the amino acid sequence coded by variant exon v6 of the human CD44 gene. The sequence of variant exon v6 as well as of the other variant

5    exons is known in the art (Screaton, GR *et al.*, Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc. Natl. Acad. Sci. U.S.A. 89: 12160-12164, 1992; Tölg, C *et al.*, Splicing choice from ten variant exons establishes CD44 variability. Nucleic Acids. Res. 21: 1225-1229, 1993; Hofmann, M. *et al.*, CD44 splice variants confer metastatic behavior in rats: homologous

10   sequences are expressed in human tumor cell lines. Cancer Res. 51: 5292-5297, 1991). A preferred antibody molecule of the invention specifically binds to peptides or polypetides having or containing the amino acid sequence SEQ ID NO:1 of the accompanying sequence listing, or an allelic variant of said sequence. Preferably, said antibody molecule has binding specificity for an epitope within said sequence. More preferably, the antibody

15   molecule specifically binds to a peptide having the amino acid sequence SEQ ID NO:2, even more preferably having the amino acid sequence SEQ ID NO:3. Such antibody molecules may be easily produced with methods known in the art (WO 95/33771, WO 97/21104), e.g. by immunising laboratory animals with chemically synthesised peptides having the aforementioned sequences, e.g. bound to a hapten, or immunising with a

20   recombinantly produced fusion protein including said sequences, and proceeding according to methods known in the art (Harlow, L D. Antibodies. Cold Spring Harbor Lab.,1988; Catty D. Antibodies. Oxford IR Press, 1988; Koopman,G. *et al.*, Activated human lymphocytes and aggressive Non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. J. Exp. Med. 177: 897-904, 1993; Heider,

25   K-H *et al.*, A human homologue of the rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. J. Cell Biol. 120: 227-233, 1993).

Preferably, an antibody molecule to be used for the  present invention is the murine

30   monoclonal antibody with the designation VFF-18 which is produced by a hybridoma cell line which has been deposited on 07 June 1994 under the accession number DSM

ACC2174 with the DSM-Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Deutschland/Germany. Also preferred are Fab, Fab', or F(ab)$_2$ fragments of said monoclonal antibody VFF-18. In another preferred embodiment, the antibody molecule is a humanised recombinant

5    antibody, wherein the complementarity determining regions (CDR's) of VFF-18 have been grafted into the respective genes of human immunoglobulin heavy and light chains.

"Complementarity determining regions" of a monoclonal antibody are understood to be those amino acid sequences involved in specific antigen binding according to Kabat, EA *et*

10    *al.*, Sequences of Proteins of Immunological Interest (5th Ed.). NIH Publication No. 91-3242. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD, 1991, in connection with Chothia and Lesk, J. Mol. Biol. 196: 901-917, 1987.

15    In another preferred embodiment, appropriate framework residues of such a CDR-grafted antibody are reverted to murine residues to improve binding affinity. From methods pertinent to the art, the experts knows how to obtain the CDR's of VFF-18, starting with the aforementioned hybridoma with the accession number DSM ACC2174, to choose and obtain appropriate human immunoglobulin genes, to graft the CDR's into these genes, to

20    modify selected framework residues, to express the CDR-grafted antibody in appropriate host cells, e.g. Chinese hamster ovary (CHO) cells, and to test the resulting recombinant antibodies for binding affinity and specificity (see e.g. literature references above). In another preferred embodiment of the invention, the antibody molecule is a recombinant antibody having the CDR's of the antibody VFF-18. Preferably, such a recombinant

25    antibody is a humanised antibody and is a complete immunoglobulin consisting of two complete light and two complete heavy chains. In another preferred embodiment of the invention, the antibody molecule is a recombinant antibody having the same idiotype as the antibody VFF-18. In another preferred embodiment of the invention, the antibody molecule is a recombinant antibody binding to the same epitope as the antibody VFF-18.

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In a particular preferred embodiment, the antibody molecule **A** is an antibody comprising light chains having the amino acid sequence SEQ ID NO:4, and heavy chains having the amino acid sequence SEQ ID NO:6. This antibody is called BIWA 4. It is a humanised version of antibody VFF-18 mentioned above, having the complementary determining

5    regions of the murine monoclonal antibody VFF-18 in a completely human framework, and human constant regions. It is therefore an antibody of very low immunogenicity in man, which is a favorable trait. However, as it has no murine framework residues to optimise antigen binding, it has a significanty lower antigen binding affinity as its parent antibody VFF-18, and therefore would not have been regarded as a good candidate for a

10   therapeutic drug. Unexpectedly, it has been found that BIWA 4, despite its poor binding affinity, has a very favorable biodistribution and tumor uptake in vivo, making it superior to other humanised versions of VFF-18 with higher binding affinity. In a further preferred embodiment, the antibody molecule **A** is an antibody comprising light chains having the amino acid sequence SEQ ID NO:8, and heavy chains having amino acid sequence SEQ

15   ID NO:6. This antibody is called BIWA 8 and has higher binding affinity than BIWA 4.


These antibodies may be produced as follows. Nucleic acid molecules coding for the light chain and the heavy chain may be synthesised chemically and enzymatically by standard methods. First, suitable oligonucleotides can be synthesized with methods known in the

20   art (e.g. Gait, MJ, Oligonucleotide Synthesis. A Practical Approach. IRL Press, Oxford, UK, 1984), which can be used to produce a synthetic gene. Methods to generate synthetic genes from oligonucleotides are known in the art (e.g. Stemmer et al. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. Gene 164(1): 49-53, 1995; Ye et al. Gene synthesis and expression in E. coli for pump, a human

25   matrix metalloproteinase. Biochem. Biophys. Res. Commun. 186(1):143-9, 1992; Hayden and Mandecki Gene synthesis by serial cloning of oligonucleotides. DNA 7(8): 571-7, 1988; Frank et al. Methods Enzymol. 154: 221-249, 1984). Preferably, the nucleic acid molecules encoding the light and heavy chains of BIWA 4 have the nucleotide sequences of SEQ ID NO:5 and SEQ ID NO:7, respectively. These sequences include sequences

30   coding for leader peptides which are cleaved by the host cell (SEQ ID NO:5: the first 60 nucleotides; SEQ ID NO:7: the first 57 nucleotides). In a further embodiment, the nucleic

acid molecules encoding the light and heavy chains of an antibody molecule according to the invention have the nucleotide sequences of SEQ ID NO:9 and SEQ ID NO:7, respectively. These nucleic acid molecules encoding the antibody heavy and light chains then may be cloned into an expression vector (either both chains in one vector molecule, or

5 each chain into a separate vector molecule), which then is introduced into a host cell. Expression vectors suitable for immunoglobulin expression in prokaryotic or eukaryotic host cells and methods of introduction of vectors into host cells are well-known in the art. In general, the immunoglobulin gene therein is in functional connection with a suitable promoter, like for example a human cytomegalovirus (CMV) promoter, hamster ubiquitin

10 promoter (WO 97/15664), or a simian virus SV40 promoter located upstream of the Ig gene. For termination of transcription, a suitable termination/polyadenylation site like that of the bovine growth hormone or SV40 may be employed. Furthermore, an enhancer sequence may be included, like the CMV or SV40 enhancer. Usually, the expression vector furthermore contains selection marker genes like the dihydrofolate reductase

15 (DHFR), glutamine synthetase, adenosine deaminase, adenylate deaminase genes, or the neomycin, bleomycin, or puromycin resistance genes. A variety of expression vectors are commercially available from companies such as Stratagene, La Jolla, CA; Invitrogen, Carlsbad, CA; Promega, Madison, WI or BD Biosciences Clontech, Palo Alto, CA. For example, expression vectors pAD-CMV1 (NCBI GenBank Accession No. A32111) or

20 pAD-CMV19 (NCBI GenBank Accession No. A32110) may be used for expression. The host cell preferably is a mamalian host cell, e.g. a COS, CHO, or BHK cell, more preferably a Chinese hamster ovary (CHO) cell, e.g. a CHO-DUKX (Urlaub and Chasin, Proc. Natl. Acad. Sci. U.S.A. 77(7): 4216-20, 1980), CHO-DG44 (Urlaub et al., Cell 33: 405-412, 1983), or CHO-K1 (ATCC CCL-61) cell. The host cell then is cultured in a

25 suitable culture medium under conditions where the antibody is produced, and the antibody is then isolated from the culture according to standard procedures. Procedures for production of antibodies from recombinant DNA in host cells and respective expression vectors are well-known in the art (see e.g. WO 94/11523, WO 97/9351, EP 0 481 790, EP 0 669 986).

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In order to link the antibody molecule **A** to the compound **B** which is toxic to cells, a linking moiety **L** is used. In the most simple case, the linking moiety **L** is a chemical bond, preferably a covalent bond which is cleaved under intracellular conditions. In one embodiment of the invention, the bond is between a sulfur atom present in the antibody

5    molecule, e.g. in the side chain of a cystein residue, and another sulfur atom present in the toxic compound. In another embodiment, the linking moiety **L** consists of one or more atoms or chemical groups. Suitable linking groups are well known in the art and include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups and esterase labile groups. Preferred are disulfide groups and thioether groups.

10

Conjugates of the antibody molecules of the invention and toxic compound can be formed using any techniques presently known or later developed. The toxic compound can be modified to yield a free amino group and then linked to the antibody molecule via an acid-labile linker, or a photolabile linker. The toxic compound can be condensed with a peptide

15    and subsequently linked to an antibody molecule to produce a peptidase-labile linker. The toxic compound can be treated to yield a primary hydroxyl group, which can be succinylated and linked to an antibody molecule to produce a conjugate that can be cleaved by intracellular esterases to liberate free drug. Most preferably, the toxic compound is treated to create a free or protected thiol group, and then one or many disulfide or thiol-

20    containing toxic compounds are covalently linked to the antibody molecule via disulfide bond(s).

For example, antibody molecules can be modified with crosslinking reagents such as N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), 4-succinimidyl-oxycarbonyl-α-

25    methyl-α-(2-pyridyldithio)-toluene (SMPT), N-succinimidyl-3-(2-pyridyldithio)-butyrate (SDPB), N-succinimidyl-4-(2-pyridyldithio)pentanoate (SPP), N-succinimidyl-5-(2-pyridyldithio)pentanoate, 2-iminothiolane, or acetylsuccinic anhydride by known methods. See, Carlsson et al, Biochem. J. 173: 723-737, 1978; Blattler et al. Biochem. 24:1517-1524, 1985; Lambert et al, Biochem. 22: 3913-3920, 1983; Klotz et al, Arch. Biochem.

30    Biophys. 96: 605, 1962; Liu et al, Biochem. 18: 690, 1979; Blakey and Thorpe, Antibody, Immunoconjugates and Radiopharmaceuticals, 1: 1-16, 1988; Worrell et al, Anti-Cancer

Drug Design 1: 179-184, 1986. In a preferred embodiment, the linker moiety is a 5-thiopentanoate or 4-thiopentanoate derived from SPP. The antibody molecule containing free or protected thiol groups thus derived is then reacted with a disulfide- or thiol-containing toxic compound to produce conjugates. The conjugates can be purified by

5  HPLC or by gel filtration.

„Toxic" is a compound that inhibits or prevents function of cells and/or causes cell destruction. Toxic compounds used for coupling may act either cytostatic or cytotoxic and lead to cell cycle arrest or cell death. These compounds may act at different stages during

10  the cell cycle, e.g. by interference with nucleic acid synthesis, inactivation of nucleic acids, or by binding to tubulin.

In a preferred embodiment, the compound $B$ present in $A(LB)_n$ which is toxic to cells is a maytansinoid, i.e. a derivative of maytansine (CAS 35846538). In a preferred

15  embodiment, it is a C-3 ester of maytansinol. Maytansinoids suitable for conjugating to antibodies for use in cancer therapy, including preparation of said maytansinoids and their linkage to antibody molecules, have been described by Chari et al. (Chari, RVJ et al., Immunoconjugates containing novel maytansinoids: promising anticancer drugs. Cancer Research 52: 127-31, 1992; Liu, C et al., Eradication of large colon tumor xenografts by

20  targeted delivery of maytansinoids. Proc. Natl. Acad. Sci. U.S.A 93: 8618-23, 1996; U.S. Patent No. 5,208,020). These maytansinoids may be used for the present invention. In a preferred embodiment, the toxic compound is $N^{2'}$-deacetyl- $N^{2'}$-(3-mercapto-1-oxopropyl)-Maytansine (CAS Number 139504-50-0), also referred to as DM1. Preferably, said maytansinoid is a maytansinol derivative linked to the antibody molecule via a disulfide

25  bridge at the C-3 position of maytansinol. In a particularly preferred embodiment, the antibody/maytansinoid conjugate may be prepared from a maytansinoid of formula

Formula (II)
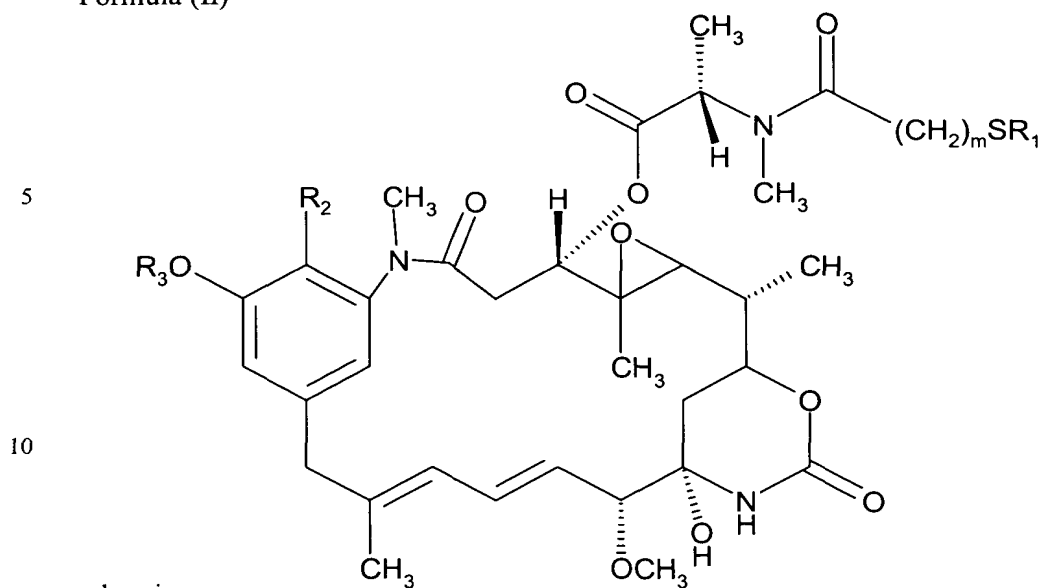


wherein

$R_1$    represents H or $SR_4$, wherein $R_4$ represents methyl, ethyl, linear alkyl, branched alkyl, cyclic alkyl, simple or substituted aryl, or heterocyclic;

$R_2$    represents Cl or H;

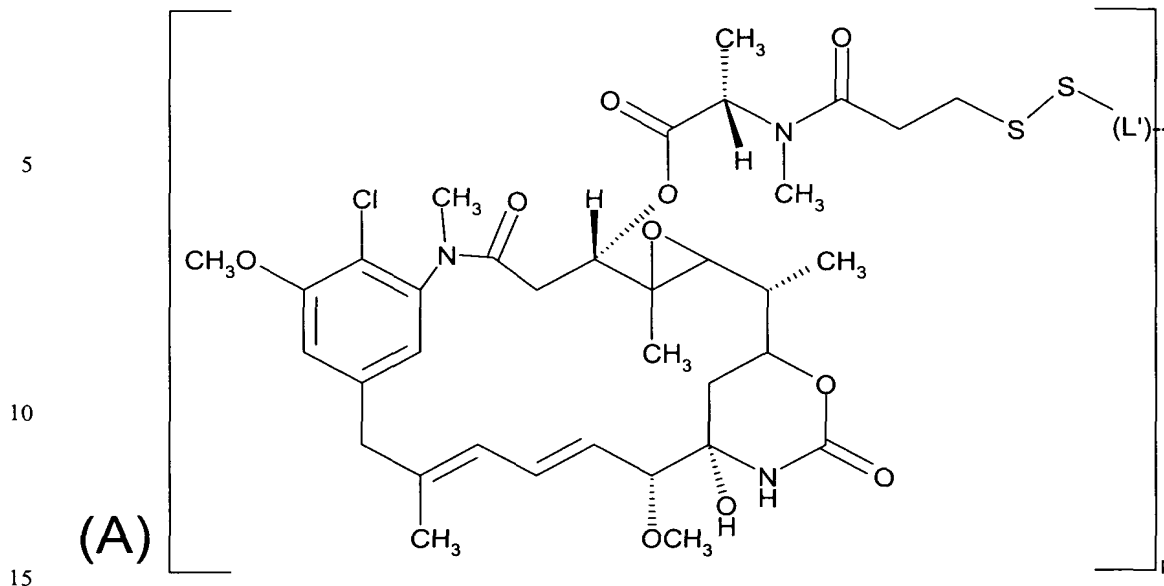$R_3$    represents H or $CH_3$; and

m    represents 1, 2, or 3.

Preferably, $R_1$ is H, $CH_3$, or $SCH_3$, $R_2$ is Cl, $R_3$ is $CH_3$, and m = 2.

The compound with $R_1$ = H, $R_2$ = Cl, $R_3$ = $CH_3$, and m = 2 is designated DM1 in the literature.

In a preferred embodiment, the compound of the invention has the formula:

(Formula III)



(A)

wherein

A is an antibody molecule which is specific for CD44, preferably specific for the variant exon v6, preferably specific for the amino acid sequence SEQ ID NO:3;

(L') is an optional linker moiety

p is a decimal number with p = 1 to 10

Preferably, p is 3 to 4, more preferably about 3.5.


Methods for preparing such maytansinoids are known in the art (see in particular U.S. 5,208,020, Example 1). Conveniently, in a first step the maytansinoid C-3 ester ansamitocin P3 may be produced by bacterial fermentation (U.S. Patent 4,356,265; U.S. Patent 4,450,234; WO 01/77360) of microorganisms belonging to the genus *Nocardia* or *Actinosynnema*, e.g. ATCC 31565, ATCC 31281. Ansamitocin P3 may be extracted from the culture using organic solvents like ethyl acetate or toluene, and further purified by adsorption chromatography using e.g. silica gel. It may then be reduced to maytansinol using LiAlH$_4$ (U.S. 4,360,462) or, as suggested more recently (WO 02/16368), LiAl(OMe)$_3$H or other LiAl or NaAl hydrids. The maytansinol may then be esterified at the C-3 position with N-methyl-L-alanine or N-methyl-L-cysteine derivatives to yield a disulfide-containing maytansinoid (U.S. 5,208,020; U.S. 5,416,064; U.S. 6,333,410), for example using dicyclohexylcarbodiimide(DCC) and catalytic amounts of zinc chloride

(U.S. 4,137,230; U.S. 4,260,609). In a preferred embodiment, the maytansinol is esterified with the compound N-methyl-N-(3-methyldithiopropanoyl)-L-alanine of formula:

5    
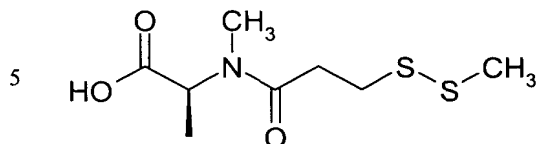
to yield the maytansinoid of Formula (II) with with $R_1 = SR_4$, $R_4 = CH_3$, $R_2 = Cl$, $R_3 = CH_3$, and $m = 2$. Manufacture of N-methyl-L-alanine or N-methyl-L-cysteine derivatives is

10   disclosed for example in U.S. 5,208,020 and WO 02/22554.


The free thiol group may then be released by cleavage of the disulfide bond with dithiothreitol (DTT), to yield e.g. DM1.


15   Upon intracellular cleavage, the free toxic compound is released. The free drug released from the compound **A(LB)$_n$** may have the formula **B-X**, wherein **X** is an atom or a chemical group, depending on the nature of the cleaving reaction. Preferably, **X** is a hydrogen atom, as for example when the linker moiety is just a covalent bond between two sulfur atoms, or a hydroxyl group. The cleavage site may also be within the linker moiety

20   if the linker moiety is a chemical group, generating free drug of formula **B-L"-X** upon cleavage, wherein **X** is an atom or a chemical group, depending on the nature of the cleaving reaction. Preferably, **X** is a hydrogen atom or a hydroxyl group. The free drug released intracellularly may also contain parts (amino acid or peptidic residues) of the antibody molecule, if the linker ist stable, but the antibody molecule is degraded.

25

In a preferred embodiment, the compound of Formula (I) is less toxic than the toxic compound **B**, **B-X** or **B-L"-X** released upon intracellular cleavage. Methods of testing cytotoxicity in vitro are known in the art (Goldmacher et al., J. Immunol. 135: 3648-3651, 1985; Goldmacher et al., J. Cell Biol. 102: 1312-1319, 1986; see also U.S. 5,208,020,

30   Example 2). Preferably, the compound (I) is 10 times or more, more preferably 100 times or more, or even 1000 times or more less toxic than the free drug released upon cleavage.

Preferably, antibody molecule/maytansinoid conjugates are those that are joined via a disulfide bond, as discussed above, that are capable of delivering maytansinoid molecules. Such cell binding conjugates are prepared by known methods such as modifying

5    monoclonal antibodies with succinimidyl pyridyl-dithiopropionate (SPDP) or pentanoate (SPP; N-succinimidyl-4-(2-pyridyldithio)pentanoate, or N-succinimidyl-5-(2-pyridyldithio)pentanoate) (Carlsson et al, 1978). The resulting thiopyridyl group is then displaced by treatment with thiol-containing maytansinoids to produce disulfide linked conjugates. Alternatively, in the case of the aryldithiomaytansinoids, the formation of the

10   antibody conjugate is effected by direct displacement of the aryl-thiol of the maytansinoid by sulfhydryl groups previously introduced into antibody molecules. Conjugates containing 1 to 10 maytansinoid drugs linked via a disulfide bridge are readily prepared by either method. In this context, it is understood that the decimal number $n$ in the formula $A(LB)_n$ is an average number as not all conjugate molecules of a given preparation may

15   have the identical integer of **LB** residues attached to the antibody molecule.

More specifically, a solution of the dithiopyridyl modified antibody at a concentration of 1 mg/ml in 0.1 M potassium phosphate buffer, at pH 7.0 containing 1 mM EDTA is treated with the thiol-containing maytansinoid (1.25 molar equivalent/dithiopyridyl group). The

20   release of pyridine-2-thione from the modified antibody is monitored spectrophotometrically at 343nm and is complete in about 30 min. The antibody-maytansinoid conjugate is purified and freed of unreacted drug and other low molecular weight material by gel filtration through a column of Sephadex G-25. The number of maytansinoids bound per antibody molecule can be determined by measuring the ratio of

25   the absorbance at 252 nm and 280 nm. An average of 1-10 maytansinoid molecules/antibody molecule can be linked via disulfide bonds by this method.

In a preferred aspect, the present invention relates to a conjugate of a CD44v6 specific antibody molecule and a maytansinoid. Herein, „CD44v6 specific" shall mean that the

30   antibody has specific binding affinity to an epitope which is present in a peptide having the amino acid sequence encoded by variant exon v6 of CD44, preferably human CD44. A

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preferred antibody molecule of the invention specifically binds to peptides or polypetides

having or containing the amino acid sequence SEQ ID NO:1 of the accompanying

sequence listing, or an allelic variant of said sequence. Preferably, said antibody molecule

has binding specificity for an epitope within said sequence. More preferably, the antibody

5    molecule specifically binds to a peptide having the amino acid sequence SEQ ID NO:2,

even more preferably having the amino acid sequence SEQ ID NO:3.


Preferably, the antibody molecule in said conjugate is the monoclonal antibody VFF-18

(DSM ACC2174) or a recombinant antibody having the complementary determining

10    regions (CDRs) of VFF-18. More preferably, the said antibody comprises light chains

having the amino acid sequence SEQ ID NO:4, or, alternatively, SEQ ID NO:8, and heavy

chains having the amino acid sequence SEQ ID NO:6.


The maytansinoid is preferably linked to the antibody by a disulfide moiety and has the

15    formula:


Formula (IV)
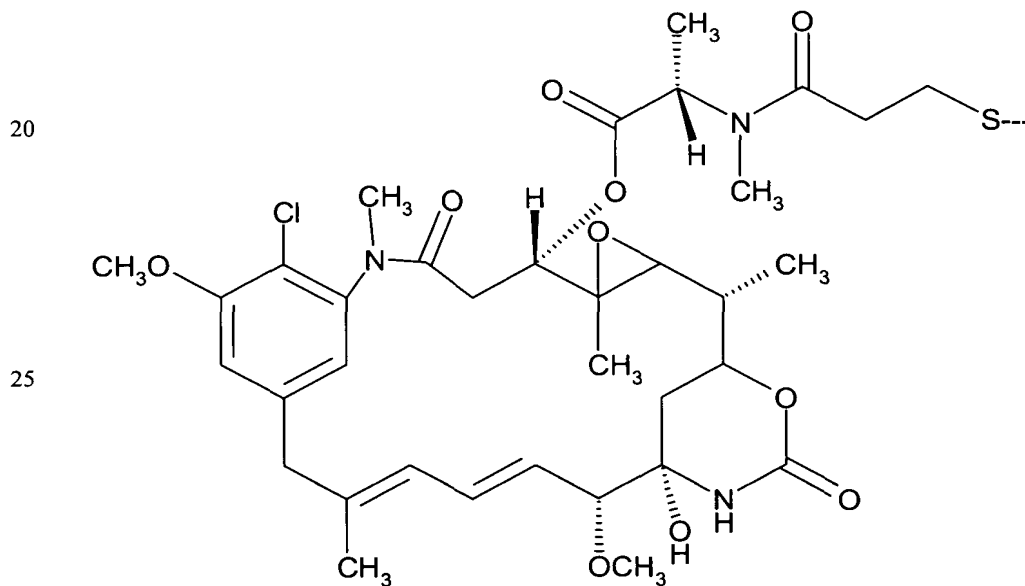


30    wherein the link to the antibody is through the sulfur atom shown in formula IV to a

second sulfur atom present in the antibody molecule. To create such a sulfur atom

available for bonding, an antibody molecule may be modified by introduction of a suitable linker as outlined above. Preferably, the maytansinoid is linked to the antibody molecule through a $-S-CH_2CH_2-CO-$, a $-S-CH_2CH_2CH_2CH_2-CO-$, or a $-S-CH(CH_3)CH_2CH_2-CO-$ group. The sulfur atom in such a linker group forms the disulfide bond with the

5     maytansinoid, while the carbonyl function may be bonded to an amino function present on the side chain of an amino acid residue of the antibody molecule.

That way, one or more maytansinoid residues may be linked to an antibody molecule. Preferably, 3 to 4 maytansinoid residues are linked to an antibody molecule.
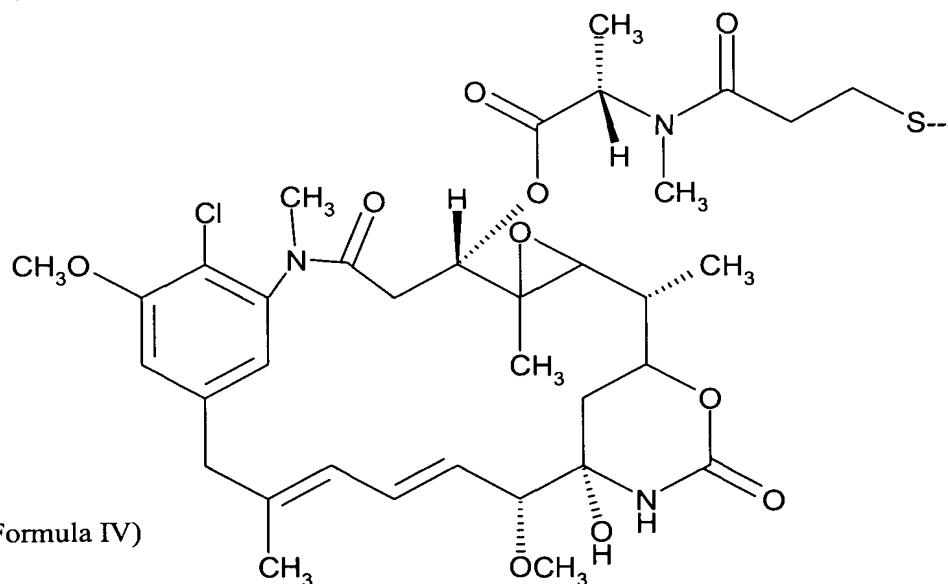
10

Most preferred is a conjugate of a CD44v6 specific antibody molecule and a maytansinoid, wherein the antibody comprises light chains having the amino acid sequence SEQ ID NO:4, and heavy chains having the amino acid sequence SEQ ID NO:6, and wherein the maytansinoid has the formula

15



20

25     (Formula IV)

and is linked to the antibody through a disulfide bond. Preferably, the linking group is $-S-CH_2CH_2CH_2CH_2-CO-$ or $-S-CH(CH_3)CH_2CH_2-CO-$, and the number of maytansinoid

30     residues bound per antibody molecule is 3 to 4.

The conjugate or compound of Formula (I), as defined herein, is preferably formulated into a pharmaceutical composition comprising such conjugate or compound, preferably together with a pharmaceutically acceptable carrier, excipient, or diluent.

5    Suitable pharmaceutically acceptable carriers, diluents, and excipients are well known and can be determined by those of skill in the art as the clinical situation warrants. In general, the conjugate may be formulated in form of a buffered aqueous solution, using a physiologically acceptable buffer like phosphate buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l $Na_2HPO_4$, 0.24 g/l $KH_2PO_4$ in distilled water, adjusted to pH 7.4 with

10   aqueous HCl), which may contain additional components for solubilisation, stabilisation, and/or conservation, e.g. serum albumin, ethylenediaminetetraacetate (EDTA), benzyl alcohol, or detergents like polyoxyethylenesorbitan monolaurate (Tween 20™). Examples of suitable carriers, diluents and/or excipients include: (1) Dulbecco's phosphate buffered saline, pH about 7.4, containing about 1 mg/ml to 25 mg/ml human serum albumin, (2)

15   0.9% saline (0.9% w/v NaCl), and (3) 5% (w/v) dextrose. The formulation may also be in form of a freeze-dried powder which may be reconstituted with water or buffer before administration. Such lyophilisates may contain an bulking agent like, for example, mannitol.

20   For clinical treatment of cancer, the compound of Formula (I) according to the invention, in particular the conjugate of a CD44v6 specific antibody molecule and a maytansinoid, will be supplied as solutions that are tested for sterility and for endotoxin levels. Examples of suitable protocols of conjugate administration are as follows. Conjugates may be given weekly for 1 to 6 weeks either as an i.v. bolus , or as a continuous infusion

25   for 5 days. Bolus doses can be given in 50 to 100 ml of isotonic saline to which 5 to 10 ml of human serum albumin has been added. Continuous infusions can be given in 250 to 500 ml of isotonic saline, to which 25 to 50 ml of human serum albumin has been added, per 24 hour period. Dosages will be 10 mg to 400 mg/m$^2$ of body surface area per application. The dose applied to the patient per administration has to be high enough to be

30   effective, but must be below the dose limiting toxicity (DLT). In general, a sufficiently well tolerated dose below DLT will be considered maximum tolerated dose (MTD). The

expert knows how to determine the MTD (Lambert, JM et al., Pharmacokinetics, in vivo stability, and toxicity of the Tumor-activated prodrug, C242-DM1, a novel colorectal cancer agent. Proceedings of the American Association of Cancer Research 39: Abs 3550, 1998). For weekly administrations, the MTD can be expected to be in the range of 50 to

5  200 mg/m$^2$. Alternatively, intervals between applications may be longer, e.g. two to four weeks, preferably three weeks. In this case, the MTD can be expected to be in the range of 100 to 300 mg/m$^2$. Alternatively, application may be in 5 daily doses, followed by a break of several weeks after which treatment may be repeated. In this case, the MTD per administration can be expected to be lower than 100 mg/m$^2$. For example, conjugates can

10  be administered as a single i.v. infusion with a rate of 3 mg/min every 21 days.

„Chemotherapeutic agent", in the context of this invention, shall mean a chemical compound which inhibits or kills growing cells and which can be used or is approved for use in the treatment of cancer. As the compound of Formula (I), in particular the

15  conjugate of a CD44v6 specific antibody molecule and a maytansinoid itself is a chemotherapeutic agent in that sense, it is understood that the present invention relates to the combination of such a compound of Formula (I) or conjugate with a second, structurally different chemotherapeutic agent. Preferably, the chemotherapeutic agent to be combined with a compound of Formula (I), or conjugate as defined above, is not itself

20  an immunoconjugate. Preferred chemotherapeutic agents for such a combination are cytostatic agents which prevent, disturb, disrupt or delay cell divison at the level of nuclear division or cell plasma division. Preferably, the chemotherapeutic agent is an anti-mitotic agent, in particular it is a spindle poison acting by interfering with microtubule function, causing mitotic arrest. Such agents may be classified in two groups, those like taxanes that

25  stabilise microtubule lattices or those, among them the vinca alcaloids, that preferentially form alternate lattice contact and polymers at microtubule ends and thus destabilise microtubules (Future Oncology 6: 1421-1456, 2002; Goodsell, The Oncologist 5: 345-346, 2000). Both types of spindle poisons are comprised in the present invention.

30  Hence, preferred chemotherapeutic agents are those which bind to tubulin. Preferred agents stabilising microtubules are taxanes, in particular docetaxel or paclitaxel, and

epothilones, in particular epothilone A, B, C, D, E, and F. Preferred agents which destabilise microtubules are vinca alcaloids, in particular vinblastine, vincristine, vindesine, vinflunine, and vinorelbine.

5  Taxanes are anti-mitotic agents isolated from yew trees of the genus *taxus* and their naturally occurring, semi-synthetically, or synthetically obtained derivatives (Future Oncology 6: 1421-1456, 2002; The Oncologist 5: 345-346, 2000). Paclitaxel (Taxol®, Suffness [Ed.], Taxol®. Science and Applications. CRC Press, Boca Raton, 1995; Wani et al., J. Am. Chem. Soc. 93: 2325, 1971; Holton et al., J. Am. Chem. Soc. 116: 1597, 1599,

10  1994; Runowicz et al., Cancer 71: 1591-1596, 1993) is an approved anti-cancer drug formulated as a non-aqueous solution (in polyoxyethylated castor oil/ethanol) intended for dilution with a suitable parenteral fluid for intravenous infusion at doses of 15-275 mg/m$^2$ applied as 1-, 6-, or 24 hour infusions. For combination therapy with another chemotherapeutic drug, an infusion at a dose of 135 or 175 mg/m$^2$ over a period of 3 or 24

15  hours is recommended. Docetaxel (Taxotere®; EP 0 253 738; U.S. 4,814,470; Mangatal et al., Tetrahedron 45: 4177, 1989; Denis et al., J. Org. Chem. 56: 6939, 1991; Burris et al., J. Clin. Oncol. 11: 950, 1993) is also an approved anti-neoplastic taxane drug. Recommended adminstration is at a dose of 60 to 100 mg/m$^2$ infused intravenously over a period of 1 week. Another taxane useful in the context of the present invention is RPR-

20  116258A (Goetz, A D et al., Proc. Am. Soc. Clin. Oncol., 20:Pt 1 (Abs 419), 2001). Other taxanes known in the art which are useful for cancer treatment may be used for the present invention as well. Such taxanes are disclosed for example in WO 01/70718, WO 01/57028, WO 01/57027, WO 01/56564, WO 01/57030, WO 01/57029, WO 01/57031, WO 01/56565, WO 01/57032, WO 01/27115, WO 01/55126, WO 00/50059, U.S. Patent

25  6,002,023, WO 99/52887, U.S. 5,998,656, WO 99/32473, U.S. Patent 5,892,063, WO 99/14209, U.S. Patent 5,763,477, WO 98/14187, WO 98/08833, WO 98/02426, U.S. Patent 5,705,508, U.S. Patent 5,703,247, WO 97/43291, WO 97/10234, WO 97/09979, EP 747 372, WO 96/21658, GB 2296239, WO 96/14308, WO 96/03394, EP 693 485, GB 2289277, WO 95/25728, WO 95/24402, WO 95/11020, EP 639 577, WO 94/27984, U.S.

30  Patent 5,367,086, WO 94/20088, WO 95/07900, WO 94/21651, WO 94/21252, WO 94/21251, WO 94/21250, EP 617 034, WO 94/17052, WO 94/17050, WO 94/25449, WO

94/15599, EP 604 910, WO 94/12484, WO 94/10997, WO 94/08984, EP 668 762, WO 94/11362, WO 94/01425, WO 93/23389, EP 534 709, EP 534 708, and EP 534 707.

Other compounds that can be used in the invention are those that act through a taxane
mechanism. Compounds that act through a taxane mechanism include compounds that
have the ability to exert microtubule-stabilizing effects and cytotoxic activity against
proliferating cells, such as tumor cells or other hyperproliferative cellular diseases. Such
compounds include, for example, epothilone compounds, such as, for example, epothilone
A, B, C, D, E, F, BMS-247550, BMS- 310705, and derivatives thereof. Epothilone
compounds are spindle poisons produced by certain myxobacteria and their naturally
occurring, semi-synthetically, or synthetically obtained derivatives. Epothilone
compounds and derivatives thereof are known in the art and are described, for example, in
U. S. Patent Nos. 6,121,029, 6,117,659, 6,096,757, 6,043,372, 5,969,145, 5,886,026, WO
97/19086, WO 98/08849, WO 98/22461, WO 98/25929, WO 98/38192, WO 99/01124,
WO 99/02514, WO 99/03848, WO 99/07692, WO 99/27890, and WO 99/28324.
Particularly preferred is epothilone B (CAS No. 152044-54-7; WO 98/25929; White et al.,
Org. Lett. 1(9): 1431-1434, 1999; Valluri et al., Org Lett 3 (23): 3607-3609, 2001;
Muhlradt et al., Cancer Res 57(16): 3344-3346, 1997; Chou et al., Proc. Natl. Acad. Sci.
U.S.A 95(16): 9642-9647, 1998; Chen et al., Proc. Ann. Meet. Am. Assoc. Cancer Res.
41: Abs 4578, 2000) which may be applied at doses between 0.3 and 3.6 mg/m$^2$, more
preferably between 0.3 and 2.5 mg/m$^2$. Also preferred is the epothilone B analogue BMS-
247550 disclosed by Yamaguchi et al., Cancer Res. 62 (2): 466-471, 2002, and Lee et al.,
Clin Cancer Res 7(5): 1429-1437, 2001. Also preferred is the epothilone A analogue
BMS-310705 (Vite et al., ACS Meeting 2002, 223rd:Orlando(MEDI 18), 2002; Mekhail et
al., Proceedings of the American Society for Clinical Oncology, 21:1(Abs 408), 2002).
Also preferred is epothilone A, the synthesis of which is described by Zhu and Panek, Org.
Lett. 2 (17): 2575-2578, 2000, and epothilone E, the synthesis of which is described by
Nicolaou et al., Bioorg. Med. Chem. 7(5): 665-697, 1999. Also preferred is epothilone D
(U.S. 6,204,388; U.S. 6,303,342; Wang et al., AACR NCI EORTC Molecular Targets and
Cancer Therapeutics 2001, October 29-November 2(Abs #781), 2001; Chou et al., Proc.

Natl. Acad. Sci. U.S.A 95(16): 9642-9647, 1998) which may be applied at a dose of 10-20 mg/m².

Vinca alcaloids are spindle poisons produced by plants of the genus *catharanthus*
5   (formerly *vinca* Linn.), in particular *catharanthus roseus*, and their naturally occurring, semi-synthetically, or synthetically obtained derivatives. Vinblastine, e.g. in the form of its sulfate salt, is an approved anti-cancer drug (Gorman et al., J. Am. Chem. Soc. 81: 4745-4754, 1959; U.S. 3,097,137; U.S. 3,225,030; Sieber et al., Cancer Treat. Rep. 60: 127, 1976; Lu and Meistrich, Cancer Res. 39: 3575, 1979; Muhtadi and Afifi, Analytical
10   Profiles of Drug Substances and Excipients 21: 611-658, Brittain (Ed.), Academic Press, San Diego, 1992). The sulfate salt may be formulated as pure substance which is dissolved in physiological saline before administration and may be applied as a intravenous bolus injection at a dose of 3 to 18.5 mg/m², preferably 5.5 to 7.5 mg/m² once weekly. Vincristine, e.g. in the form of its sulfate, is also an approved anti-cancer drug
15   (Neus et al., J. Am. Chem. Soc. 86: 1440, 1964; Sieber et al., Cancer Treat. Rep. 60: 127, 1976; Owellen and Donigian, J. Med. Chem. 15: 894, 1972; Burns, Anal. Prof. Drug Subs. 1: 463-480, 1972). The sulfate may be formulated as a solid preparation containing an equal amount of lactose as an excipient and dissolved in isotonic saline before administration. 1 to 2 mg/m² of the drug may be applied per week as an intravenous bolus
20   application. Vindesine is a synthetic derivative of vinblastine and also an approved chemotherapeutic agent (DE 2415980; Burnett et al., J. Med. Chem. 21: 88, 1978; Owellen et al., Cancer Res. 37: 2603, 1977; Krivit et al., Cancer Chemother. Pharmacol. 2: 267, 1979). Vindesine sulfate may be formulated as a powder formulation with mannitol as an excipient (at a ratio of 1:5) and dissolved in water or isotonic saline before
25   administration. 2-4 mg/m² may be applied as a weekly intravenous bolus injection. Vinorelbine is a semi-synthetic vinca alcaloid also approved for cancer therapy (U.S. 4,307,100; Mangeney et al., Tetrahedron 35: 2175, 1979; Rahmani et al., Cancer Res. 47: 5796-5799, 1987; Marty et al., Nouv. Rev. Fr. Hematol. 31: 77-84, 1989). It may be formulated as a vinorelbine bis[(R,R)-tartrat] in water at a concentration of 10 mg/ml,
30   diluted with isotonic saline or 5% glucose solution before administration, and intravenously infused at a dose of 20 to 30 mg/m² per week. Vinflunine (CAS 162652-95-

1; Decosterc et al., Anti-Cancer Drugs 10(6): 537-543, 1999) and navelbine (Van-den-Berge et al., Anticancer Res. 13(1): 273-277, 1993) may also be used for the present invention. In the art, further vinca alcaloids are known which may be used in connection with the present invention (WO 99/62912; WO 98/45301; U.S. 5,369,111; U.S. 5,888,537;

5    U.S. 5,891,724; 5,676,978; U.S. 4,096,148).

Further microtubule-destabilising agents to be used in the context of the present invention are 5,6-dihydroindolo[2,1-a]isoquinoline derivatives (Goldbrunner et al., J. Med. Chem. 40(22): 3524-3533, 1997). Particularly suitable is combretastatin A4-phosphate (Horsman

10    et al., Proc. Annu. Meet. Am. Assoc. Cancer Res. 39: Abs 1142, 1998), and its derivatives like hydroxphenastatin (Pettit et al., J. Med. Chem. 43(14): 2731-2737, 2000), or AVE 8062 (Ohsumi et al., J. Med. Chem. 41(16): 3022, 1998). Spongistatins like spongistatin 1, 2, 3, 4, 5, 6, 7, 8, or 9, may be also used (EP 0 608 111; EP 0 632 042; EP 0 634 414). Another tubulin antagonist for use in the present invention is E-7010 (CAS 141430-65-1;

15    Hoshi and Castaner, Drugs Future 18(11): 995-996, 1993). Other tubulin antagonists which may be used in the context of the present invention are dolastatins like cemadotin hydrochloride (Mross et al., Onkologie 19(6): 490-495, 1996). Mivobulin isethionate may also be used (De-Ines et al., Cancer Res. 54(1): 75-84, 1994).

20    The compound of formula (I), in particular the conjugate of a CD44v6 specific antibody molecule and a maytansinoid, may be combined with other chemotherapeutic agents like cryptophycins, camptothecins (in particular, camptothecin, topotecan, irinotecan, 9-aminocamptothecin), or epipodophyllotoxins (in particular, etoposide, or teniposide). Also to be used in connection with the present invention are anthracyclines like

25    doxorubicin and daunorubicin. Furthermore, antibiotics like dactinomycin, plicamycin, mitomycin, bleomycin, and idarubicin may be used. Alkylating agents like cyclophosphamide, mechlorethamine, melphalan, chlorambucil, procarbazine, dacarbazine, altretamine, platinum compounds (in particular, cisplatin, carboplatin, oxaliplatin, iproplatin, ormaplatin, tetraplatin), or nitrosureas like carmustine, lomustine,

30    or semustine may be used as well. Also comprised are methotrexate, purine antagonists like mercaptopurine, thioguanine, fludarabine phosphate, cladribine, pentostatin, or

pyrimidine antagonists like fluorouracil, doxifluridine (5'-deoxy-5-fluorouridine), capecitabine, cytarabine, or azacytidine. In a further preferred embodiment, the chemotherapeutic agent is capecitabine (N4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine; Ishikawa et al., Biol Pharm Bull. 21(7):713-7, 1998).

5

In the context of this invention, „in combination with" shall mean that the compound of Formula (I), in particular the conjugate of a CD44v6 specific antibody molecule and a maytansinoid, and the chemotherapeutic agent are administered to the patient in a regimen wherein the patient may profit from the beneficial effect of such a combination. In

10 particular, both drugs are applied to the patient in temporal proximity. In a preferred embodiment, both drugs are applied to the patient within four weeks (28 days). More preferably, both drugs are administered within two weeks (14 days), more preferred within one week (7 days). In a preferred embodiment, the two drugs are administered within two or three days. In another preferred embodiment, the two drugs are administered at the

15 same day, i.e. within 24 hours. In another embodiment, the two drugs are applied within four hours, or two hours, or within one hour. In another embodiment, the two drugs are administered in parallel, i.e. at the same time, or the two administrations are overlapping in time. For example, they may be infused at the same time, or the infusions may be overlapping in time. If the two drugs are administered at the same time, they may be

20 formulated together in one single pharmaceutical preparation, or they may be mixed together immediately before administration from two different pharmaceutical preparations, for example by dissolving or diluting into one single infusion solution. In another embodiment, the two drugs are administered separately, i.e. as two independent pharmaceutical compositions. In one preferred embodiment, administration of the two

25 drugs is in a way that tumor cells within the body of the patient are exposed to effective amounts of both drugs at the same time. In another preferred embodiment, effective amounts of both drugs are present at the site of the tumor at the same time. In another preferred embodiment, effective amounts of both drugs are present in the body of the patient at the same time. The present invention also embraces the use of further agents,

30 which are administered in addition to the combination as defined. This could be, for example, one or more further chemotherapeutic agent(s). It could also be one or more

agent(s) applied to prevent, suppress, or ameliorate unwanted side effects of any of the other drugs given. For example, a cytokine stimulating proliferation of leukocytes may be applied to ameliorate the effects of leukopenia or neutropenia.

5    Dose, route of administration, application scheme, repetition and duration of treatment will in general depend on the nature of the disease (type, grade, and stage of the tumor etc.) and the patient (constitution, age, gender etc.), and will be determined by the medical expert responsible for the treatment. With respect to the possible doses for the components of the disclosed combination which are described above, it is clear that the medical expert

10   responsible for the treatment will carefully monitor whether any dose-limiting toxicity or other severe side effects occur and undertake the necessary steps to manage those.

The present invention is of particular advantage for the treatment of squameous cell carcinomas expressing CD44 antigen, preferably CD44v6. It is in particular suitable for

15   head and neck squameous cell carcinoma, esophagus squameous cell carcinoma, lung squameous cell carcinoma, skin squameous cell carcinoma, or cervix squameous cell carcinoma. Furthermore, it is of particular advantage for the treatment of adenocarcinomas expressing CD44 antigen, perferably CD44v6. It is in particular suitable for breast adenocarcinoma, lung adenocarcinoma, pancreas adenocarcinoma, colon

20   adenocarcinoma, or stomach adenocarcinoma. Besides treatment of clinically apparent malignant disease, therapeutic application according to the invention may be particularly advantageous as an adjuvant to surgical intervention, to treat minimal residual disease.

In a further aspect, the present invention relates to a pharmaceutical composition

25   comprising a compound $A(LB)_n$ or conjugate as defined herein, together with a chemotherapeutic agent as defined herein, and optionally further comprising one or more pharmaceutically acceptable carrier(s), diluent(s), or excipient(s).

In a further embodiment, the present invention relates to a kit comprising, in separate

30   pharmaceutical compositions, a compound $A(LB)_n$ as defined before, in particular a

conjugate of a CD44v6 specific antibody molecule and a maytansinoid, and a chemotherapeutic agent as herein defined.

In a further embodiment, the present invention relates to the use of a chemotherapeutic

5    agent for the preparation of a pharmaceutical composition for the treatment of cancer, wherein said chemotherapeutic agent is used or is for use in combination with a compound of Formula $A(LB)_n$ as herein defined, in particular a conjugate of a CD44v6 specific antibody molecule and a maytansinoid. Preferably, the chemotherapeutic agent is a taxane, an epothilone, a vinca alcaloid, or another tubulin antagonist, a platinum

10   compound, a camptothecin, a cryptophycin, a dolastatin, an epipodophyllotoxin, an alkylating agent, an purine antagonist, a pyrimidine antagonist, or a DNA intercalator. In preferred embodiments, the chemotherapeutic agent is docetaxel, paclitaxel, RPR-116258A, epothilone A, B, C, D, E, or F, BMS-247550, BMS-310705, vinblastine, vindesine, vincristine, vinorelbine, combretastatin A4-phosphate, hydroxphenastatin, AVE

15   8062, spongistatin 1, 2, 3, 4, 5, 6, 7, 8, or 9, E-7010, dolastatin, cemadotin hydrochloride, mivobulin isethionate, cryptophycin, camptothecin, topotecan, irinotecan, 9-aminocamptothecin, cisplatin, carboplatin, oxaliplatin, iproplatin, ormaplatin, tetraplatin, etoposide, teniposide, doxorubicin, daunorubicin, dactinomycin, plicamycin, mitomycin, bleomycin, idarubicin, cyclophosphamide, mechlorethamine, melphalan, chlorambucil,

20   procarbazine, dacarbazine, altretamine, carmustine, lomustine, semustine, methotrexate, mercaptopurine, thioguanine, fludarabine phosphate, cladribine, pentostatin, fluorouracil, cytarabine, or azacytidine.

In a further aspect, the present invention relates to a method of treatment of cancer,

25   wherein an effective amount of a chemotherapeutic agent, as defined herein, is administered to a patient in need thereof in combination with a with a compound of Formula $A(LB)_n$. as herein defined, in particular a conjugate of a CD44v6 specific antibody molecule and a maytansinoid.

30

The present invention is further described in the following examples which are provided for illustrative purposes only and are not to be construed as limiting. Indeed, other variants of the invention will be readily apparent to one of ordinary skill in the art.

5    All publications and patents cited herein are incorporated by reference in their entireties.

## Examples

### 1. Manufacturing and characterisation of BIWI 1

10   **1.1. Manufacturing of BIWI 1**

Humanised recombinant antibodies BIWA 4 and BIWA 8 which have binding specificity for an epitope within SEQ ID NO:1 were linked to the maytansinoid DM1 as described below. The conjugate of BIWA 4 with DM1 was designated BIWI 1.

15   **Generation of stably transfected cell lines.** The genes coding for the light and heavy chains of BIWA 4, SEQ ID NO:5 and SEQ ID NO:7, were ligated into expression vector pAD-CMV1 (WO92/01055; NCBI GenBank Accession No. A32111) or pAD-CMV19 (NCBI GenBank Accession No. A32110). In the second antibody BIWA 8, the light chain was coded by a gene having SEQ ID NO:9, while the heavy chain was the same as in

20   BIWA 4. Stably transfected cell lines were generated by electroporation as follows. CHO DUX/57ss (dhfr negative mutant of Chinese Hamster Ovary cells, adapted for serum free suspension culture) were used. After trypsinisation and inactivation of trypsin with RPMI-10 (90% RPMI 1640, 10% heat inactivated fetal calf serum), cells were washed once with RPMI-0 (RPMI 1640 without serum), and $1 \times 10^7$ cells were resuspended in 0.8 ml RPMI-

25   0. After addition of the linearised DNA (20 µg per plasmid; cotransfection of vectors coding for light and heavy chain) the cells were electroporated using a Hoefer Electroporator under the following conditions: 1080 µF, 320 V, 1000 msec, 1 pulse. Cells were allowed to stand for 5 min, and were then diluted to 12500 cells/ml and 2500 cells/ml in alfa-MEM 10d (90% MEM alfa without ribonucleosides and without

30   desoxyribonucleosides (GIBCO BRL), 10% heat inactivated dialysed fetal calf serum). The cells were seeded into 96 well microtiter plates (200 µl/well, corresponding to 2500

and 500 cells/well respectively). Clones appeared after 10 days. Only the plates with 500 cells/well were followed up (3-6 clones/well). After 14-15 days, supernatants from each well were tested in a κ/γ ELISA. 53 clones were seeded in 12 well plates in alfa-MEM 10d. After 3-6 days (depending on the confluency of the cells) supernatants were tested

5 again in the κ/γ ELISA (serial dilutions) and quantitated using a human IgG1 standard. Cells were frozen and stored in liquid nitrogen. IgG contents of the 53 clones ranged from 12 - 417 ng/ml. 10 clones with the highest expression level were selected and subcloned as follows: Cells of each clone were seeded into 96 well microtiter plates with densities of 1 and 5 cells/well in 100µl/well alpha-MEM 10d (1 plate for each clone and each density).

10 Eight days later supernatants were diluted 1:2 and 100 µl of this dilution tested in the κ/γ ELISA and quantitated using a BIWA 4 preparation as standard. Five subclones of each clone were transferred to 12 well plates. The IgG content ranged from 1.3 - 908 ng/ml. Fourteen clones with the highest expression level (384 - 908 ng/ml) were used for amplification with methotrexate as follows: Clones were initially cultured in 25 cm$^2$

15 flasks containing alfa-MEM 10d with 20, 50 and 100 nM methotrexate. After the outgrowth of clones the supernatants were tested in the κ/γ ELISA. In subsequent rounds of amplification the methotrexate concentration was raised up to 2000 nM. Initially the highest expression level ranged from 10.5-14.8 µg/ml (clone A31/100, 100 nM methotrexate). Further amplification with a methotrexate concentration of 500 nM gave

20 an expression of 19-20 µg/ml (A31/500).

**Purification of antibody.** Antibody was purified from cell culture supernatant as follows. Antibody containing tissue culture supernatant was applied onto a 5 ml protein A sepharose column with a flow rate of 80-90 ml/h at 4 °C. After washing with 50 ml

25 binding buffer (0.1 M sodium phosphate pH 7.5), the Ig fraction was eluted with elution buffer (0.1 M glycine-HCl pH 2.7). Absorption at 280 nm was monitored.

**Modification of BIWA 4 with SPP to form BIWA 4-SS-Py.** BIWA 4 was supplied in liquid form at a concentration of 5 mg/mL in a PBS formulation containing Tween 20.

30 Prior to coupling of DM1 to the MAb the Tween 20 was removed. The MAb solution (40 mL) was diluted 15-fold with 25 mM MES buffer, pH 5.6, containing 50 mM NaCl (MES

buffer) and then loaded onto a column (12.5 mL) of Sepharose S equilibrated in MES

buffer (flow rate: 100 cm/hr). The column was washed with 10 column volumes of MES

buffer. The antibody was eluted with MES buffer containing 400 mM NaCl. The

antibody solution was dialysed against 50 mM potassium phosphate buffer, pH 6.5

5    containing 50 mM NaCl and 2 mM EDTA (Buffer A). The BIWA 4 antibody was

modified using SPP ((2-Pyridyl)-5-dithiopentanoic acid N-hydroxy succinimid ester) to

introduce dithiopyridyl groups. The MAb in Buffer A (185 mg, 8 mg/mL) was modified

with a 7-fold molar excess of SPP in EtOH (5% v/v of MAb solution). The reaction

proceeded for 90 minutes at ambient temperature. The reaction mixture was then

10    subjected to gel filtration chromatography through Sephadex G25F (2.6 x 31.5 cm

column, 167 mL) equilibrated in Buffer A. MAb-containing fractions were pooled and the

degree of modification was determined by measuring the absorbance at 280 nm and the

change in absorbance at 343 nm caused by the release of 2-mercaptopyridine by the

addition of DTT. The concentration of released 2-mercaptopyridine was calculated using

15    an $\varepsilon_{343\,nm}$ of 8080 $M^{-1}cm^{-1}$, and the concentration of MAb was calculated using an $\varepsilon_{280\,nm}$

of 224,000 $M^{-1}cm^{-1}$ after the absorbance at 280 nm has been corrected for the contribution

from 2-mercaptopyridine. (2-mercaptopyridine $A_{280\,nm} = A_{343\,nm}$ x 5100/8080). Recovery

of the MAb was 99.6% with 5.5 releasable 2-mercaptopyridine groups linked per MAb

molecule.

20

**Conjugation of BIWA 4-SS-Py with DM1.** The above modified MAb (184 mg) in

Buffer A was conjugated at 2.5 mg MAb/mL using a 1.7-fold molar excess of DM1 over

releasable 2-mercaptopyridine groups. DM1 was added in DMA (3% v/v of MAb

solution) and the reaction mixture was incubated at ambient temperature for 29 hours. The

25    conjugate was then isolated by gel filtration chromatography on a column of Sephacryl

S300 HR equilibrated in PBS (5 x 50 cm column, 980 mL, flow rate of 10 cm/hr). The

conjugate eluted as a single peak at the position of monomeric MAb with a small amount

of protein eluting earlier. Fractions were assayed for the number of DM1 molecules linked

per MAb molecule. (Linked DM1 molecules were determined by measuring the

30    absorbance at both 252 nm and 280 nm). Based on the results, fractions representing 63-

77% of the column volume were pooled. The DM1/MAb ratio in the pooled solution was

found to be 3.1 and the yield of conjugated BIWA 4 was 75% based on starting MAb. The conjugate, BIWI 1, was evaluated by SDS-PAGE performed under non-reducing conditions and found to be composed primarily of a monomer species (>95%) with a minor amount (<5%) of dimeric conjugate.

## 1.2. Analysis of *in vitro* binding of BIWI 1.

The binding of BIWA 4 antibody and BIWI 1 conjugate to antigen-positive FaDu cells was determined. Cells ($1\text{-}2 \times 10^{-5}$) were incubated in 96-well plates with varying concentrations of antibody or conjugate on ice for 1 hour. The test article was washed from the plate and FITC-labeled anti-human IgG was added and the incubation on ice was continued in the dark for 1 hour. After washing, the cells were fixed with 1% paraformaldehyde and analyzed on a fluorescence activated cell sorter (FACS). BIWA 4 antibody binds with an apparent $K_D$ of $1 \times 10^{-9}$ M and BIWI 1 binds with an apparent $K_D$ of $1.8 \times 10^{-9}$ M. Thus, conjugation with DM1 alters the binding affinity of the antibody only slightly if at all.

## 1.3. *In vitro* cytotoxicity of BIWI 1

For determination of viable cells the Cell Titer 96® AQ$_{ueous}$ non-radioactive cell proliferation assay (Promega) was used. Five thousand cells per well were seeded into 96-well plates in 90 µl medium without phenole red. Cells were allowed to settle down for 1 to 3 h and then serial dilutions of the immunoconjugate in 10 µl PBS were added. Cells without immunoconjugate served as negative control. Cells were incubated for 4 days at 37°C in a humified 5% $CO_2$ atmosphere and then 20 µl MTS/ PMS were added according to the manufacturer's recommendation. After additional 1 to 4 h incubation at 37°C the absorbance at 490 nm was recorded using an ELISA plate reader. For each cell line triplicates were analyzed. The percentage of the surviving cell fraction and the IC50 value were calculated using the GraphPad Prism® (Version 3.0) software.

The *in vitro* cytotoxicity of BIWI 1 was evaluated using the antigen-positive cell lines A431 and FaDu, and the antigen-negative cell line A459. Cells were exposed to different concentrations of BIWI 1 for 4 days, then stained with MTS/ PMS and assayed on an

ELISA plate reader. The surviving fractions of cells were then calculated using the GraphPad Prism® software package. The results are shown in Figure 1. BIWI 1 was effective in killing the antigen-positive A431 cells with an $IC_{50}$ of about 7.6 x $10^{-8}$ M and the second antigen-positive cell line, FaDu, with an $IC_{50}$ of about 2.4 x $10^{-8}$ M. The

5    antigen-negative cell line, A549, was effected by the conjugate with a surviving fraction of 50% at the highest concentration of BIWI 1 tested (5 x $10^{-7}$ M). These results show that BIWI 1 is only slightly more cytotoxic against antigen-positive cells than antigen-negative cells *in vitro*. For comparison, another DM1-antibody conjugate has been shown to be at least 1000 fold more cytotoxic against antigen-positive cell as compared to antigen-

10   negative cells (Chari *et al.*, 1992).

## 2. Efficacy studies in nude mice

### 2. 1. Xenograft models

15   *In vivo* anti-tumor efficacy of BIWI 1 was tested in three nude mouse xenograft models applying antigen-positive human tumors: A431 (ATCC # CRL 1555; epidermoid carcinoma of the vulva), FaDu (ATCC # HTB 43; squamous cell carcinoma of the pharynx), and MDA-MB 453 (ATCC # HTB-131; breast carcinoma). The cells were received from ATCC and cultured in RPMI1640 medium containing 10% fetal calf serum

20   and supplements. $1 \times 10^6$ tumors cells were transplanted subcutaneously into the right flank of 6 week old female NMRI-nu/nu mice. Tumor growth was monitored by measuring tumor size. A tumor response was rated as complete response when the tumor completely disappeared at any time after start of treatment. The response was rated as partial response when the tumor volume decreased after treatment but thereafter started regrowing. The

25   tolerability of the treatment was monitored by measuring mouse weight during the observation period.

### 2.2. BIWI 1 monotherapy in A431 xenografted nude mice

Mice were randomised into the following treatment groups (treatment/initial mean tumor

30   volume/tumor volume range/number of mice):

33

Group 1:    Control (PBS)/ $185 \pm 217$ mm$^3$ / $19 - 424$ mm$^3$ / 5 mice.

Group 2:    BIWA 4 (21 mg/kg/d) / $133 \pm 115$ mm$^3$ / $42 - 302$ mm$^3$ / 5 mice.

Group 3:    BIWI 1 (2.1 mg/kg/d) / $107 \pm 63$ mm$^3$ / $42 - 205$ mm$^3$ / 5 mice.

Group 4:    BIWI 1 (7 mg/kg/d) / $132 \pm 73$ mm$^3$ / $42 - 205$ mm$^3$ / 5 mice.

5          Group 5:    BIWI 1 (21 mg/kg/d) / $107 \pm 63$ mm$^3$ / $42 - 205$ mm$^3$ / 5 mice.


Groups of 5 mice were treated with 2.1 mg/kg/d BIWI 1, 7 mg/kg/d BIWI 1, 21 mg/kg/d

BIWI 1, and 21 mg/kg/d control antibody, respectively. Treatment consisted of i.v.

injections of BIWI 1 given on five consecutive days, starting at day 1. The average tumor

10    volume of each group during the observation period is shown in Figure 2. Tumors treated

with control antibody showed similar growth as untreated tumors, the tumor volume

doubling time was approximately 5 days. In animals treated either with 7 mg/kg/d BIWI 1

or 21 mg/kg/d BIWI 1 all tumors responded completely and disappeared around day 17.

No tumor regrowth was observed until the end of the observation period (day 134).

15    Tumors treated with 2.1 mg/kg/d responded completely in 3/5 cases with no tumor

regrowth until day 134. The remaining 2 tumors showed a partial response but ultimately

regrew. These results show that BIWI 1 induces a dose-dependent anti-tumor response in

A431 xenografted nude mice, with complete and long-lasting responses from 2.1 mg/kg/d

BIWI 1 to 21 mg/kg/d BIWI 1. Unconjugated control antibody shows no anti-tumor

20    effect. See Figure 2.


### 2.3.    BIWI 1 monotherapy in FaDu xenografted nude mice

Mice were randomised into the following treatment groups (treatment/initial mean tumor

volume/tumor volume range/number of mice):

25

Group 1:    Control (PBS)/ $142 \pm 82$ mm$^3$ / $34 - 268$ mm$^3$ / 8 mice.

Group 2:    BIWA 4 (21 mg/kg/d) / $134 \pm 86$ mm$^3$ / $42 - 268$ mm$^3$ / 6 mice.

Group 3:    BIWI 1 (2.1 mg/kg/d) / $149 \pm 96$ mm$^3$ / $50 - 268$ mm$^3$ / 6 mice.

Group 4:    BIWI 1 (7 mg/kg/d) / $132 \pm 97$ mm$^3$ / $42 - 268$ mm$^3$ / 6 mice.

30         Group 5:    BIWI 1 (21 mg/kg/d) / $129 \pm 74$ mm$^3$ / $50 - 231$ mm$^3$ / 6 mice.

Groups of 6 mice were treated with 2.1 mg/kg/d BIWI 1, 7 mg/kg/d BIWI 1, 21 mg/kg/d BIWI 1, and 21 mg/kg/d control antibody, respectively. Treatment consisted of i.v. injections of BIWI 1 given on five consecutive days, starting at day 1. The average tumor volume of each group during the observation period is shown in Figure 3. Tumors treated

5    with control antibody and 2.1 mg/kg/d BIWI 1 showed similar growth as untreated tumors, the tumor volume doubling time was approximately 5 days. In animals treated with 21 mg/kg/d BIWI 1 all tumors responded completely and disappeared around day 24. No tumor regrowth was observed until the end of the observation period (day 107). Tumors treated with 7 mg/kg/d BIWI 1 responded completely in 1/6 cases, 3/6 tumors showed

10   partial responses. The remaining 2 tumors grew similar to untreated control tumors. These results show that BIWI 1 induces a dose-dependent anti-tumor response in FaDu xenografted nude mice, with complete and long-lasting responses from 7 mg/kg/d BIWI 1 to 21 mg/kg/d BIWI 1. Unconjugated control antibody shows no anti-tumor effect. See Figure 3.

15

**2.4. BIWI 1 monotherapy in MDA-MB 453 xenografted mice**

Groups of 6 mice were treated with 6.25 mg/kg BIWI 1, 12.5 mg/kg BIWI 1, and 25 mg/kg BIWI 1, respectively. Treatment consisted of i.v. injections of BIWI 1 given weekly for four weeks. The average tumor size at start of treatment was 246 +/- 79 mm$^3$ (PBS), 216

20   +/- 85 mm$^3$ (6.25 mg/kg BIWI 1), 188 +/- 79 mm$^3$ (12.5 mg/kg BIWI 1), and 207 +/- 96 mm$^3$ (25 mg/kg BIWI 1), respectively. The average tumor volume of each group during the observation period is shown in Figure 4. The initial tumor volume doubling time of the control tumors was approximately 5 days. In animals treated with 25 mg/kg BIWI 1 all tumors responded completely and disappeared around day 22 after start of treatment. No

25   tumor regrowth was observed until the end of the observation period (day 64). Tumors treated with 12.5 mg/kg or 6.25 mg/kg responded completely in 5/6 cases in each dose group, and 4 animals of each group stayed tumor free until the end of the experiment. These results show that BIWI 1 induces anti-tumor responses in MDA-MB 453 xenografted nude mice when given once a week over a period of four weeks, with

30   complete and long-lasting responses from 6.25 mg/kg BIWI 1 to 25 mg/kg BIWI 1. See Figure 4.

## 2.5. Tolerability of BIWI 1 monotherapy in nude mice

The tolerability of BIWI 1 monotherapy was determined by monitoring mouse weight during the whole duration of the experiment in the 2 models. The maximum observed

5      average weight loss per group was 6% in FaDu xenografted mice treated with 21 mg/kg/d BIWI 1 (Figure 5). The weight loss started around day 3 of treatment and lasted until day 10, thereafter animals regained weight and behaved similar as control animals. In all other dose groups weight loss was similar to vehicle control (PBS). An average weight loss of 6% or less in all treatment groups indicates good tolerability of BIWI 1 treatment at the

10      given doses in nude mice. As BIWI 1 does not cross-react with mouse CD44v6, only antigen-independent effects such as toxicity caused by free DM1 can be monitored in this experiment. See Figure 5.


## 2.6. BIWI 1 combination therapy with paclitaxel in the FaDu xenograft model

15      Mice were randomised into the following treatment groups (treatment/initial mean tumor volume/tumor volume range/number of mice):

         Group 1:      Control (PBS)/ 197 mm$^3$ / 65 – 402 mm$^3$ / 8 mice

         Group 2:      Paclitaxel / 182 mm$^3$ / 65 – 302 mm$^3$ / 8 mice

         Group 3:      BIWI 1 / 208 mm$^3$ / 65 – 382 mm$^3$ / 8 mice

20          Group 4:      BIWI 1 + paclitaxel/ 159 mm$^3$ / 79 – 335 mm$^3$ / 7 mice


Treatment consisted either of five i.v. injections of BIWI 1 (7 mg/kg corresponding to 100 µg/kg DM1) at day 1, 3, 5, 8, 10, or of six i.p. injections of paclitaxel (10 mg/kg) at day 2, 4, 6, 9, 11, 13, respectively, or a combination thereof. Control animals were treated with

25      PBS.


The results of the experiments are summarised in Table 1. The mean relative tumor volumes during treatment are shown in Figure 6, individual tumor volumes are shown in Figure 7. Monotherapy with paclitaxel resulted in a tumor growth delay compared to PBS

30      treated animals. BIWI 1 monotherapy resulted in a partial response in 3/ 8 animals which showed a clear delay in tumor growth compared to PBS-treated animals. In the

combination group all tumors responded to treatment and showed clearly delayed tumor

growth compared to control tumors. One tumor completely dissappeared during treatment

and the animal remained tumor free until the end of the observation period. These

experiments demonstrate a markedly increased anti-tumor efficacy of a combination of

5     BIWI 1 with paclitaxel compared to the respective monotherapies, indicating a synergistic

effect of the combination therapy.

**Table 1:** Initial tumor doubling time, growth delay factors and number of complete tumor

regressions for different treatment groups

10

| Treatment Group | Initial Tumor Doubling Time (d) | Growth Delay Factor | Complete Regression |
|---|---|---|---|
| Control | 4 | n.a. | 0/8 |
| Paclitaxel | 7 | 0.75 | 0/8 |
| BIWI 1 | 7 | 0.75 | 0/8 |
| Combination BIWI 1 + Paclitaxel | 39 | 8.75 | 1/7 |

See also Figures 6 and 7.

The tolerability of the treatment was determined by monitoring mouse weight during the

15    whole duration of the experiment. The maximum observed average weight loss per group

was less than 5% in in the combination group. In all other dose groups basically no weight

loss upon treatment was observed. An average weight loss of less than 5% indicates good

tolerability of the treatment at the given doses in nude mice.

20    **2.7.    BIWI 1 combination therapy with docetaxel and cisplatin in the FaDu**

**xenograft model**

Experiments were performed using nude mice xenografted subcutaneously with the human

head and neck squamous cell carcinoma FaDu. To determine the efficacy of BIWI 1 as

single drug given i.v., different dose levels were tested in a once weekly x 4 schedule. To

25    assess possible antagonistic, additive or supraadditive effects in combination with

chemotherapy, non-curative doses of BIWI 1 were combined with non-curative doses of

docetaxel or cisplatin. The efficacy of these combinations was compared to those of the

respective monotherapies by i) analysing the tumor volumes over time and ii) by determining the time for tumors to reach a certain size (tumor growth delay).

BIWI 1 batch (2.95 mg/ml protein, 53.6 µg/ml DM1, 3.7 molecules DM1/molecule Ab)
5    was diluted in PBS according to the indicated DM1 concentrations. The dilution was stored at +4°C during the whole treatment period.. The antibody solution was injected into the tail vein with an injection volume of 200 µl for a 25 g mouse. In the control groups without BIWI 1 treatment animals were injected i.v. with PBS. Docetaxel (Taxotere®, Aventis, 10 mg/ml) was diluted in 0.9% NaCl according to the indicated concentrations.
10   The solution was injected into the tail vein with an injection volume of 200 µl for a 25 g mouse. Cisplatin (Cisplatin "Ebewe", 1 mg/ml) was diluted in 0.9% NaCl according to the indicated concentrations. The solution was injected into the tail vein with an injection volume of 200 µl for a 25 g mouse.

15   Tumor sizes and body weights were recorded 2-3 times per week. Tumor sizes were measured with a calliper (length and width) and the volume was calculated according to the following formula: volume = (length) x (width)$^2$ x ($\pi$ / 6). The tolerability of the treatment was monitored by measuring mouse weight during the whole observation period. Animals were sacrificed when tumors reached a volume of more than 1600 mm$^3$ or when
20   animals showed a weight loss of more than 15%. For calculation of the mean tumor volumes per group, the last values of tumor size for individual animals were carried forward in case that these animals had to be sacrificed due to large tumor burden before the whole group was terminated. In addition to the absolute tumor volume in mm$^3$, the relative tumor volumes (RTV) were calculated as follows:

25

RTV = (tumor volume at day x) / (tumor volume at start of treatment) x 100
For the evaluation of treatment efficacy the following parameters were calculated:

- T/C (%) = (mean RTV of treated group) / (mean RTV of control group) x 100
30   - T4T (tumor quadrupling time) = time until the mean RTV reaches 400%
- GDF$_4$ (growth delay factor) = T4T treated / T4T control.

38

In addition, for some experiments, the mean time for tumors to reach 8 times (T8T) their initial sizes were also calculated. The growth delay factor (GDF$_8$) was then calculated accordingly. Percentages of mean relative tumor volumes of treated versus control groups (T/C) were calculated on the days indicated. Percentages of mean relative tumor volumes of treated versus control groups (T/C) were calculated on the days indicated. For statistical evaluation of combination therapies exact Wilcoxon tests were used. Tolerability was assessed by monitoring body weight changes.

To assess the efficacy of BIWI 1 in combination with the taxane docetaxel (Taxotere®), non-curative doses of both drugs were tested as single agents or in combination in nude mice xenografted with FaDu$_{DD}$ tumors. In pilot experiments a dose of 3 mg/kg docetaxel (q7dx4) was determined to be non-curative in this model. BIWI 1 was used at the non-curative dose of 200 μg/kg DM 1 and at a somewhat higher dose (300 μg/kg DM1).

Mice were randomised into the treatment groups and parameters for evaluating treatment efficacy are shown in Table 2. The control had a T4T of about 7 days. Monotherapy with docetaxel showed moderate efficacy in tumor growth retardation (T/C 56%, GDF$_4$: 1.9). The effect was somewhat more pronounced in the two BIWI 1 monotherapy treatment groups with T/C 41-30% and GDF$_4$: 2.8-4.4. Complete tumor regression could be observed in 1/8 cases with the higher BIWI 1 dose. In contrast, tumor growth was markedly inhibited in both combination treatments (T/C 3-2%), with none of the tumors reaching a relative tumor volume of 400% during the observation period of 95 days (GDF$_4$ > 13.3). Combination of docetaxel with the lower dose of BIWI 1 resulted in 7/8 complete tumor regressions, while in the combination group with the higher BIWI 1 dose all tumors disappeared. These results indicate a supraadditive effect of the combination treatments in comparison to the respective monotherapies. Treatment was well tolerated in all groups, except for a slight unexplained weight loss (approx. -5%) in the docetaxel monotherapy group.

**Table 2:** Combination of BIWI 1 with docetaxel in nude mice xenografted with FaDu$_{DD}$ tumors: initial tumor volumes (TV) and parameters for evaluation of treatment efficacy

(for definition of the parameters see Section 4.4). Treatment schedule: once weekly for four weeks (q7dx4); 8 animals per group. The day for which T/C was evaluated is indicated.

| Groups | Initial TV mean (mm³) | Initial TV range (mm³) | T/C (d 16) | T4T (d) | GDF₄ |
|---|---|---|---|---|---|
| Control (PBS) | 166 | 113-268 | 100% | 7.2 | 1.0 |
| BIWI 1 200 μg/kg DM1 | 157 | 79-335 | 41% | 20.1 | 2.8 |
| BIWI 1 300 μg/kg DM1 | 183 | 65-382 | 30% | 31.1 | 4.4 |
| Docetaxel 3 mg/kg | 193 | 79-302 | 56% | 13.5 | 1.9 |
| BIWI 1 200 μg/kg DM1 + docetaxel 3 mg/kg | 160 | 92-268 | 3% | >95 | >13.3 |
| BIWI 1 300 μg/kg DM1 + docetaxel 3 mg/kg | 200 | 113-424 | 2% | >95 | >13.3 |

5

A different set of experiments was done with cisplatin. In a pilot experiment, a dose of 4 mg/kg cisplatin administered i.v. four times in weekly intervals was determined to be non-curative in this model, and also represents the maximum tolerated dose in the mouse strain used. This dose of cisplatin was combined with BIWI 1 at the non-curative doses of 200

10 μg/kg DM1 and 300 μg/kg DM1 already used in the previous experiment. Mice were randomised into the treatment groups and parameters for evaluating treatment efficacy are shown in Table 3. The control had a T4T of about 11 days. Monotherapy with BIWI 1 showed moderate efficacy at both dose levels (T/C 59-27%, GDF₄: 2.2-2.3), while cisplatin as single agent was ineffective (T/C 100%, GDF₄: 1.1). In contrast, combination

15 of cisplatin with BIWI 1 showed a clear therapeutic advantage with GDF₄ of 4 in the group with the lower BIWI 1 dose and >6.8 in the group with the higher BIWI 1 dose. In the latter combination group 2/8 complete tumor regressions could be observed. These results indicate a supraadditive effect of the combination treatments in comparison to the respective monotherapies. Treatment was well tolerated in all groups.

20

**Table 3:** Combination of BIWI 1 with cisplatin in nude mice xenografted with FaDu_DD tumors: initial tumor volumes (TV) and parameters for evaluation of treatment efficacy.

Treatment schedule: once weekly for four weeks (q7dx4); 8 animals per group. The day for which T/C was evaluated is indicated.

| Groups | Initial TV mean (mm³) | Initial TV range (mm³) | T/C (d 19) | T4T (d) | GDF₄ |
|---|---|---|---|---|---|
| Control (PBS) | 183 | 79-268 | 100% | 10.7 | 1.0 |
| BIWI 1 200 µg/kg DM1 | 237 | 113-382 | 59% | 23.2 | 2.2 |
| BIWI 1 300 µg/kg DM1 | 178 | 113-268 | 27% | 24.8 | 2.3 |
| Cisplatin 4 mg/kg | 218 | 132-268 | 100% | 11.3 | 1.1 |
| BIWI 1 200 µg/kg DM1 + cisplatin 4 mg/kg | 239 | 132-302 | 16% | 42.7 | 4.0 |
| BIWI 1 300 µg/kg DM1 + cisplatin 4 mg/kg | 236 | 113-382 | 3% | >72.3 | >6.8 |

**2.8. BIWI 1 combination therapy with doxorubicin or docetaxel in a human breast carcinoma model xenograft model**

Similar experiments as outlined under Section 2.6 were undertaken in a human breast carcinoma xenograft model. Experiments were performed using nude mice xenografted subcutaneously with the human breast carcinoma cell line MDA-MB-453. To determine the efficacy of BIWI 1 as single drug given i.v., different dose levels were tested in a once weekly x 4 schedule. To assess possible antagonistic, additive or supraadditive effects in combination with chemotherapy, non-curative doses of BIWI 1 were combined with non-curative doses of different cytostatics. The efficacy of these combinations was compared to those of the respective monotherapies by i) analysing the tumor volumes over time and ii) by determining the time for tumors to reach a certain size (tumor growth delay).

BIWI 1 batch (protein concentration 2-3 mg/ml, DM1 concentration 30-50 µg/ml, 3.3-3.7 molecules DM1 per molecule antibody) was diluted in PBS according to the indicated DM1 concentrations. The dilutions were prepared freshly for each application or stored at +4°C during the whole treatment period. According to the amount of BIWI 1 needed 50-500 µl of the antibody solution were injected into the tail vein. BIWI 1 dose levels are

41

indicated in μg DM1 per kg body weight (μg/kg DM1). Doxorubicin (Doxorubicin "Ebewe" ®, 2 mg/ml) was diluted in PBS. 200 μl of solutions with different concentrations were injected into the tail vein. Docetaxel (Taxotere®, Aventis, 10 mg/ml) was diluted in 0.9% NaCl. 200 μl of solutions with different concentrations were injected into the tail

5  vein. For combination therapies, BIWI 1 was injected approximately 4 hours prior to the cytostatic drug.

The xenograft model was handled slightly different as outlined under Section 2.1. Briefly, $1 \times 10^7$ MDA-MB-453 cells (ATCC No. HTB-131) were injected into the mammary fat pad

10  of female NMRI nu/nu mice. After reaching a volume of approximately 1000 mm³ tumors were excised, cut into 4 x 4 mm pieces and passaged further subcutaneously. Tumor fragments were cryoconserved in 90% RPMI 1640 / 10% dimethylsulfoxide (slow freezing process down to -80°C, storage in liquid nitrogen). For further passaging, frozen tumor fragments were thawed, washed 3 times in PBS and implanted subcutaneously into the

15  right and left flanks of a 4-5 female NMRI nu/nu mice. Tumors of these mice were then used to prepare the animals for a therapy experiment. Tumors were excised with a size of approximately 10 mm in diameter, cut into 2 x 2 mm pieces and implanted subcutaneously into the right flank of 6-8 week old female NMRI nu/nu mice. Approximately twice as many animals as needed for the experiment were prepared. When tumors reached a size of

20  5-10 mm in diameter (10-14 days after implantation), animals were randomised and treatments were started. Animals were obtained from Harlan (Germany) or M&B (Denmark). Tumor sizes and body weights were recorded as described in Section 2.7.

In pilot experiments, a dose of 6 mg/kg, doxorubicin (q7dx4) was determined to be non-

25  curative in this model. Since it was possible to cure 4/6 of the tumor bearing animals with a BIWI 1 dose of 100 μg/kg DM1, q7dx4, lower doses of the antibody conjugate (75 and 50 μg/kg DM1) were used in the combination experiment to assure non-curative treatment with BIWI 1 alone. Mice were randomised into the treatment groups and parameters for evaluating treatment efficacy are shown in Table 4. The control group, as well as the

30  animals treated with a BIWI 1 dose of 50 μg/kg DM1 or 6 mg/kg doxorubicin had a T4T of about 8 days. In the group treated with BIWI 1 at 75 μg/kg DM1 a T4T of 13 days was

observed. Combination of BIWI 1 at a dose of 50 µg/kg DM1 with doxorubicin showed a small increase in efficacy over the respective monotherapies (T4T: 11 days). However when BIWI 1 at 75 µg/kg DM1 was combined with doxorubicin the T4T was 43 days, showing a supra-additive therapeutic advantage compared to the respective monotherapies (GDF$_4$ of 5.3 compared to no delay of the doxorubicin treatment and GDF$_4$ of 1.6 of the BIWI 1 (75 µg/kg DM1) monotherapy). Treatment was well tolerated in all groups. Only two animals in the doxorubicin monotherapy group and one animal in the combination group with 75 µg/kg DM1 showed transient weight loss of more than 12%.

**Table 4:** Combination of BIWI 1 with doxorubicin in nude mice xenografted with MDA-MB-453 tumors: initial tumor volumes (TV) and parameters for evaluation of treatement efficacy (for definition of the parameters see above).

Treatment schedule: once weekly for four weeks (q7dx4); 6 animals per group. The day for which T/C was evaluated is indicated.

| Groups | Initial TV mean (mm³) | Initial TV range (mm³) | T/C (d13) | T4T (d) | GDF$_4$ |
|---|---|---|---|---|---|
| Control (PBS) | 244 | 151-424 | 100 % | 8.0 | 1.0 |
| BIWI 1 50 µg/kg DM1 | 261 | 180-302 | 68 % | 8.0 | 1.0 |
| BIWI 1 75 µg/kg DM1 | 272 | 205-382 | 59 % | 13.0 | 1.6 |
| Doxorubicin 6 mg/kg | 249 | 132-424 | 66 % | 7.8 | 1.0 |
| BIWI 1 50 µg/kg DM1 + doxorubicin 6 mg/kg | 274 | 180-424 | 65 % | 10.7 | 1.3 |
| BIWI 1 75 µg/kg DM1 + doxorubicin 6 mg/kg | 293 | 180-424 | 29 % | 42.6 | 5.3 |

In a second set of experiments, non-curative doses of docetaxel, which had been determined before, were combined with BIWI 1. Parameters for evaluating treatment efficacy are shown in Table 5. No difference between the control group and the animals treated with 3 mg/kg docetaxel could be seen during the whole treatment period (T4T 11-9 days). In the two other monotherapy groups (6 mg/kg docetaxel and BIWI 1) a moderate

tumor growth delay at the end of the treatment period could be observed (T/C: 75-71%, T4T of 14 days compared to a T4T of 11 days of the control, corresponding to a $GDF_4$ of 1.3). In contrast, both combination treatments showed an increased tumor growth retardation when compared to the respective monotherapies. BIWI 1 combined with 6

5 mg/kg docetaxel had a T4T of 37 days (T/C: 19%) compared to 14 days of the monotherapies. A similar effect could be observed when 3 mg/kg docetaxel was combined with BIWI 1 (T4T of 32 days, T/C: 30%). In both combination experiments, comparison of the growth delay factors of the combination treatments and the monotherapies suggests a supraadditive effect. BIWI 1 combined with 6 mg/kg docetaxel

10 gave a $GDF_4$ of 3.3 compared to 1.3 of the respective monotherapies. In the other combination group with 3 mg/kg docetaxel ($GDF_4$ of 2.9) the monotherapy with docetaxel was by itself ineffective ($GDF_4$: 0.8) and treatment with BIWI 1 alone gave a $GDF_4$ of 1.3. Treatment was well tolerated in all groups.

15 **Table 5:** Combination of BIWI 1 with docetaxel in nude mice xenografted with MDA-MB-453 tumors: initial tumor volumes (TV) and parameters for evaluation of treatment efficacy.

Treatment schedule: once weekly for four weeks (q7dx4); 8 animals per group. The day

20 for which T/C was evaluated is indicated.

| Groups | Initial TV mean (mm³) | Initial TV range (mm³) | T/C (d16) | T4T (d) | GDF₄ |
|---|---|---|---|---|---|
| Control (0.9% NaCl) | 239 | 179-329 | 100 % | 11.0 | 1.0 |
| BIWI 1 75 µg/kg DM1 | 235 | 147-299 | 75 % | 14.1 | 1.3 |
| Docetaxel 6 mg/kg | 253 | 179-306 | 71 % | 13.9 | 1.3 |
| Docetaxel 3 mg/kg | 288 | 122-586 | 102 % | 9.0 | 0.8 |
| BIWI 1 75 µg/kg DM1 + docetaxel 6 mg/kg | 327 | 169-486 | 19 % | 36.8 | 3.3 |
| BIWI 1 75 µg/kg DM1 + docetaxel 3 mg/kg | 280 | 192-377 | 30 % | 31.7 | 2.9 |

44

## 2.9 BIWI 1 combination therapy with capecitabine in a human breast carcinoma model xenograft model

Similar experiments as outlined under Section 2.6 were undertaken in a human breast carcinoma xenograft model, wherein BIWI 1 therapy was combined with the 5'deoxy-5-fluorouridine prodrug capecitabine (Xeloda®). The xenograft model MDA-MB-453 was handled as in Section 2.8.

BIWI 1 solution (protein concentration 2.95 mg/ml, 53.6 µg DM1/ml, 3.7 molecules DM1/molecule antibody) was diluted in PBS to a DM1 concentration of 75 µg/kg body weight. The dilution was stored at +4°C during the whole treatment period. The antibody solution was injected into the tail vein with an injection volume of 200 µl for a 25 g mouse. In the control groups without BIWI 1 treatment animals were injected i.v. with PBS. BIWI 1 was given i.v. into the tail vein at a dose of 75 µg DM1/kg once weekly, for three weeks. Capecitabine (150 mg tablets, Roche) was given intragastrically (by gavage needle, as a suspension in water) at a dose of 375 or 500 mg/kg during three weeks on 5 consecutive days per week.

The efficacy of the combinations was compared to those of the respective monotherapies as outlined in Section 2.8. To assess the efficacy of BIWI 1 in combination with capecitabine, non-curative doses of both drugs were tested as single agents or in combination in nude mice xenografted with MDA-MB-453 tumors. In previous experiments a BIWI 1 dose of 75 µg/kg DM1 was shown to be non-curative. For capecitabine, doses of 500 and 750 mg/kg, (q1dx5, 4 cycles) were determined to be non-curative, while a dose of 250 mg/kg was completely ineffective.

For the combinations experiments BIWI 1 with 75 µg/kg DM1 was combined with 375 or 500 mg/kg capecitabine. Mice were randomised into the treatment groups shown in Table 6. The control had a T4T of about 10 days. The different monotherapies showed moderate efficacy in tumor growth retardation with growth delay factors (GDF$_4$) of 2.6 for BIWI 1, 1.3 for the lower dose of capecitabine and 2.9 for the higher dose of capecitabine. No complete regressions were seen. When BIWI 1 was combined with 375 mg/kg or 500

mg/kg capecitabine the growth delay factors increased to 4.5 and 5.3 respectively and T/C at day 25 was 4% and 3% respectively. In both combination groups one complete regression could be observed.

5 **Table 6:** Combination of BIWI 1 with capecitabine in nude mice xenografted with MDA-MB-453 tumors: initial tumor volumes (TV) and parameters for evaluation of treatment efficacy.

Treatment schedules:

10 BIWI 1: once weekly for three weeks (q7dx3); Capecitabine: daily 5 times per week for three weeks (q1d5x, 3 cycles).

8 animals per group. In the groups treated with 375 mg/kg capecitabine as monotherapy and in combination with BIWI 1 only 7 animals could be evaluated (death not related to therapy or tumor growth).

15 The day for which T/C was evaluated is indicated.

| Groups | Initial TV mean (mm³) | Initial TV range (mm³) | T/C (d25) | T4T (d) | GDF₄ |
|---|---|---|---|---|---|
| Control (PBS/H₂0) | 139 | 84-180 | 100 % | 10.3 | 1.0 |
| BIWI 1 75 μg/kg DM1 | 132 | 58-212 | 31% | 26.9 | 2.6 |
| Capecitabine 500 mg/kg | 130 | 78-173 | 23% | 30.0 | 2.9 |
| Capecitabine 375 mg/kg | 130 | 82-227 | 58% | 13.8 | 1.3 |
| BIWI 1 75 μg/kg DM1 + Capecitabine 500 mg/kg | 141 | 89-204 | 3% | 54.7 | 5.3 |
| BIWI 1 75 μg/kg DM1 + Capecitabine 375 mg/kg | 133 | 80-233 | 4% | 46.2 | 4.5 |

Combination of BIWI 1 at 75 μg/kg DM1 given once weekly for 3 weeks with capecitabine at 375 or 500 mg/kg given 5 times weekly for 3 weeks, showed a significant
20 increase of the therapeutic effect from day 7 on, when comparing tumor sizes to those of the respective monotherapies. Comparison of the tumor growth delay factors indicates a

synergistic effect of BIWI 1 and capecitabine in this combination setting. All of the treatments were well tolerated without any significant weight loss of the animals.

**2.10 BIWI 1 combination therapy with paclitaxel and doxorubicin in a human breast**
5    **carcinoma model xenograft model**

Similar experiments as outlined under Section 2.6 were undertaken in a human breast carcinoma xenograft model, wherein BIWI 1 therapy was combined with either the taxane paclitaxel or the anthracycline doxorubicine. BIWI was given i.v. into the tail vein at doses of 50 µg DM1/kg/day or 100 µg DM1/kg/day for five consecutive days. Paclitaxel
10    (Taxol[®]) was given i.v. at 20 or 10 mg/kg, doxorubicin (Adriamycin[®]) at 6 or 4 mg/kg only on the first day of the treatment period, in each case 4 hours after BIWI 1 treatment. In mice bearing breast carcinoma xenografts with a volume of approximately 100 mm$^3$. BIWI 1 monotherapy was efficacious at a dose level of 100 µg DM1/kg, showing complete regression in in all (12/12) tumors. A dose level of 50 µg DM1/kg resulted in 2/12
15    complete tumor regressions. When BIWI at 50 µg DM1/kg was combined with paclitaxel at 20 or 10 mg/kg, a significant increase of the therapeutic effect was observed (Percentage of median relative tumor volumes of treated versus control groups = T/C of 0% for 20 mg paclitaxel /kg and 9 % for 10 mg paclitaxel /kg, respectively) in comparison to the respective monotherapies (T/C 19 % for BIWI 1, 13 % for 20 mg paclitaxel/kg, and 50 %
20    for 10 mg paclitaxel/kg). All treatments were well tolerated without any significant weight loss of the animals. Similar results were obtained with doxorubicin at the higher dose level, albeit associated with a somewhat higher toxicity.